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ON THE BRIGHT LINE SPECTRUM OF NOVA HERCULIS

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The present paper contains a qualitative interpretation of the main features observed by the authors and by others in the bright line spectrum of Nova Herculis. Like other novæ, Nova Herculis has shown a continuous rise in excitation since it attained maximum; until at the present time (January 29, 1936) it shows a spectrum typical of the so-called "nebular stage." For other novæ this general behavior has been described,[1] and the detailed changes for Nova Herculis need not be discussed here. It may be noted, however, that the forbidden lines of ionized iron, typical of novæ that develop slowly, were observed in the spectrum.[2]

Perhaps the most interesting feature of Nova Herculis is its duplicity, first observed by Kuiper[3] on July 4, 1935. Although this is not the first nova for which apparent multiplicity has been observed,[4] it is of especial interest because of the possibility of identifying the two components with features of the emission spectrum. There can be little doubt that the red and violet components of the doubled emission lines correspond respectively to the fainter and brighter components of the nova observed visually. Before substantiating the statement, we shall trace the history of the two components, as revealed by the bright line spectrum.

An examination of the spectrograms taken at Harvard, and those published by Merrill[5] and by McLaughlin,[6] shows that the bright lines of ionized iron in the green, and other lines excepting hydrogen, first displayed definite duplicity shortly after maximum. The doubling was visible on December 25, and conspicuous on December 26, 1934. Doubling of the bright lines had already been suspected[7] from the Harvard plates of December 20, two days before maximum; but between December 20 and December 25, the contrast shown by the bright lines was so weak, because of the brightness of the continuous background, that their structure was difficult to observe. The lines of hydrogen did not show the effect of duplicity as markedly as the other bright lines until early in March, whereas the forbidden lines of neutral oxygen showed it from their first appearance on December 25, 1934.

Until the middle of March the red component of the doubled bright lines was generally as strong as, or stronger than, the violet component. At about that time it began to weaken, particularly for the forbidden lines [OI]. For hydrogen the effect was delayed until the end of March, being detectable in Hα on Bobrovnikoff's spectrum[8] of March 30, and on that of April 2, reproduced by Merrill.[9] By April 5, Wyse[10] states that "all the other emission lines were displaced -400 km./sec., although the strongest ones showed traces of very faint, diffuse components on the red side of the normal positions." This was soon after the beginning of the great drop, which continued until the star had faded to the thirteenth magnitude on about the first of May. Wyse states: "The red components of the hydrogen lines disappeared rather suddenly about the end of April." McLaughlin,[11] describing a series of observations overlapping the previous, states: "As the nova faded, the red components of the emission bands weakened relatively to the violet components. With the subsequent brightening, the violet components alone were seen on May 18, but later the red components appeared and increased toward equality with the violet ones. During July and August the ratio V/R was approximately 1.5, as compared with 5 about June 1."

The nova had repeatedly been examined by Kuiper for duplicity, and was first seen to be double on July 4, with a difference of magnitude[12] of $0^m.4$. If we make the extremely probable assumption that the red and violet components of the bright lines refer respectively to the fainter and brighter components observed visually, we can see why the duplicity could not have been observed earlier. On March 31, April 27 and June 10, 1935, under good seeing conditions, Kuiper examined the nova and found it to be single.[3] On March 31, before the great minimum, the integrated brightness of the continuous background in the photographic region was about half a magnitude greater than that of all the bright lines in that region. In a recent investigation we have shown[13] that most of the light of the continuous spectrum originates in the "photosphere" of the central star. Therefore a star, brighter than the components combined, must have existed between them, and, with so small a separation, would have prevented their discovery. Furthermore, many of the strong double bright lines, notably Hα, the strongest of them all, had a wide underlying band, of intensity comparable to that of the two components. Thus it is not surprising that the duplicity was not observed on or before March 31. On April 27, as noted by Wyse, the bright lines were almost all single, so that apparently only one component could have been observed. Likewise on June 10, the violet components only were observed by Belorizky,[14] so that only one component could have been seen on that date. During July and August, from McLaughlin's rough estimate of the ratio 1.5 of the violet to the red component in the spectrum, a magni-

tude difference of $0^m.44$ can be computed for the visual components, in close agreement with Kuiper's visual estimate of $0^m.4$. A similar computation for the beginning of June yields a difference of $1^m.75$ between the components, which would have precluded a discovery of the duplicity. The difference of magnitude seems to have persisted with but little change, until October, 1935, according to the observations made by van Biesbroeck.[15]

There can be little doubt as to the nature of the two bodies thus observed visually and spectroscopically. They are not themselves the sources of the energy they radiate, because in the early stages the intensity of their spectral lines follows the irregular changes in brightness of the integrated light of the nova, light which resides mostly in the continuous spectrum. Inspection of the Harvard spectrograms shows that up to the middle of March the bright components of the lines remained practically equal in intensity, both following roughly the fluctuations of the light curve. This result could only be achieved by an indeed remarkable synchronization of two light sources. It seems more likely that the two bodies derive their light from the central star. Their density must be low, because they gave the forbidden lines of neutral oxygen almost from the first; and because they have given a typically nebular spectrum ever since the great minimum, after the continuous background (which we regard as coming mostly from the central star) had faded very considerably. The velocity differences of the two components of the bright lines increased greatly[5,8] between December, 1934 and May, 1935, and apparently a further increase had taken place[10] when the two components appeared again in July, 1935. The finite widths of the bright components can be interpreted as the result of expansion in the discrete masses, the rate of which seems, from Merrill's measures,[5] to have increased somewhat with the time. Both the increasing separation and the increasing width of the lines could be interpreted either as a result of acceleration under radiation pressure[16] or in terms of a change in the effective center of the radiation in the masses, each particle of which is assumed to be moving radially away from the star with a velocity which, for the different particles, is proportional to the distance. The final choice between these alternatives will depend on the correlation of the acceleration with brightness or with time, after the minimum. The two bodies must therefore be considered to have low density, and therefore probably low mass, and an external source of luminosity. They must have considerable size, because of the very probable expansion indicated by the finite width of the two emission components, and because they must subtend an appreciable solid angle at the star in order to reradiate as large a fraction of its light as they do. The above arguments indicate that the two bodies are in no sense stellar.

The two discrete masses just described must have originated very early

in the outburst, since duplicity of the lines was already suspected before the maximum; and as forbidden lines of neutral oxygen appeared immediately after maximum (indicating that the density of matter and dilution of radiation had already become very low), it is not improbable that the expulsion of these masses from the central star occurred at the very beginning of the rise to the first maximum, and was the immediate result of the disturbance that initiated the nova outburst. The visual observations of Kuiper and van Biesbroeck indicate that the two masses are separating at a rate not inconsistent with an origin at the star in December, 1934. Since both the radial velocity and the transverse velocity of the components can be observed, they must have been ejected at a considerable angle to the line of sight.

It is clear from a detailed study of the spectra that we are concerned with both absorption and emission from more than one level, as has also been pointed out by Lindblad and Öhman.[17] In the early stages of the nova we do not consider that the process just discussed is the principal one in the production of the permitted bright lines. In a previous paper we have discussed observations of the continuous spectrum and (qualitatively) of the absorption lines,[13] and have concluded that the continuous spectrum is produced in the atmosphere of the star, which atmosphere consists of matter continuously ejected from the surface. In such an atmosphere the absorption lines would be produced at very high levels, and would show velocity shifts corresponding to the speeds with which the atoms flowed outward through the "photosphere."

The permitted and forbidden bright lines must be discussed separately. In all stages of the nova, the forbidden lines can be produced in but two places: in the two discrete masses discussed above; and in the outer portions of the radially symmetrical atmosphere, which may or may not extend as far as the two ejected masses. Evidence for emission in the forbidden lines from the extended atmosphere is seen in the intensity of the light between the two components of the forbidden bright lines throughout all the early stages of the nova. Quantitative measures of this intensity in the later stages are not available, but even at that time we may expect that it will be appreciable. The relative intensities of the light coming from the atmosphere and from the discrete masses should then be a measure of the relative amounts of matter involved. This ratio cannot be more than one fourth and may be very much less. This would indicate for the combined mass of the ejected masses a minimum value of the order of 10^{29} grams.

The permitted bright lines are formed in a different manner from the forbidden lines. The discrete masses never predominate in their radiation, as is shown by the fact that their duplicity, though definite, is not conspicuous. These conclusions are based upon unpublished microphoto-

metric measures of the Harvard plates, as well as on the published data of Lindblad and Öhman, McLaughlin, Merrill, and Bobrovnikoff. Most of the intensity of the permitted lines must be produced in the atmosphere of the nova, and the problem is to determine at what level the emission occurs. There seems to be no escape from the conclusion that the absorption lines are generally formed above the broad emission lines. This view seems first to have been reached by Belopolsky[18] for Nova Aurigae, although he was severely criticized for it by Vogel.[19] Belopolsky's conclusion is, however, fully substantiated by Sayer's investigation of the hydrogen absorption and emission in the spectrum of Nova Aquilae.[20] For Nova Herculis, Lindblad and Öhman,[17] describing the structure of the H and K lines, state: "The broad emission to the red side of the absorption probably corresponds to the deepest level, for simplicity we will call it Emission A. Its apparent displacement toward the red, compared with the normal position of the line, is probably entirely caused by the one-sided absorption in the higher levels, which cuts off the broad emission A on the side of short wave-lengths." Referring to the bright emission, which we should regard as coming from the approaching mass, they state: "The emission H_2 and K_2 . . . which must correspond to a very high level in the gaseous envelope, gets gradually *apparently* displaced toward the red." The same conclusion concerning the absorption overlying the emission lines has been drawn by the authors from the microphotometer tracings of the Harvard spectrograms of Nova Herculis.

The formation of absorption lines at high levels might be accounted for on the thin shell theory, except that there is no explanation for the persistence of the absorption for any length of time. The theory of continuous ejection, with its other advantages, provides an equally good interpretation without encountering this difficulty. We visualize the continuously ejected atmosphere as maintaining a density gradient proportional to the inverse square of the radius. As we have shown,[18] in all the upper layers of such an atmosphere there will be a great excess of ultra-violet radiation, as compared with the Planck energy distribution. This conclusion is based on the assumption of a mass absorption coefficient depending only on the temperature and electron pressure at any point. At increasingly higher levels in the atmosphere (composed mostly of hydrogen as we suppose that of the nova to be) the absorption coefficient beyond the limit of the Lyman series will increase rapidly as the temperature falls to a point where we might normally expect a small amount of neutral hydrogen to exist, if local thermodynamic equilibrium were even approximately maintained. Because of this high absorption, hydrogen will continue to be completely ionized in higher levels of the atmosphere, until we reach a point where the excess radiation beyond the limit of the Lyman series has been transformed into radiation of longer wave-length. Much of this

light will be concentrated into bright hydrogen lines, particularly Lyman α; effectively a dilution process is taking place. But above this layer we should expect fairly normal atmospheric conditions to set in again, except for the excess of radiation in Lyman α, and between Lyman α and the limit of the Lyman series. The excess radiation at Lyman α will keep the hydrogen atoms of the upper layers in a state to absorb the Balmer series, while the bright Balmer lines are emitted in the lower layers by the process just described.

The excess radiation between Lyman α and the limit of the Lyman series will maintain the emission lines of elements other than hydrogen, the excitation potentials of which are less than 13.54 volts. In the early post-maximum stages, radiations requiring higher excitation were not observed, and only began to exist toward the end of March, when the total brightness of the nova had begun to decrease, and the process of continuous ejection, as we have pictured it, to break down.

Summary.—The two components of Nova Herculis observed visually can be related to conspicuous features of the bright line spectrum, and appear to be two gaseous masses of low density, ejected from the central star, probably very early in the outburst.

The strong absorption lines seen in the early post-maximum stages are considered to be formed at higher levels than the corresponding broad bright lines, in a deep atmosphere built up by the continuous ejection of material from the surface of the original star. It is the latter process that produces the continuous background, while the doubled bright lines arise in the two ejected nebulous masses.

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TISSUE CULTURES OF SPERMATOPHYTES*

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Introduction.—The literature concerning the growth of tissues of the higher plants has been reviewed by White[2] so recently that it seems unnecessary to repeat it here. Suffice it to say that after a long series of unsuccessful attempts White[3] developed a technique by which he was able to secure continuous growth of tomato roots in nutrient solutions. However, in one sense White's results were incomplete for they did not produce buds and so never grew into complete plants. This point should not be labored, for true "tissue" cultures would be grown from tissues and not from organs, and it might be thought that White's cultures in which one organ continued to develop indefinitely were nearer true tissue cultures than if they had grown into complete plants. Admitting this possibility, it still has seemed desirable to be able to grow small bits of seed plants and to secure from them development into complete plants. The present investigation was made with that end in view.

Methods.—Culture media. The solution developed by White[3] was used in these trials. The following salts were used as indicated:

Salt	Millimols
$Ca(NO_3)_2$	0.60
$MgSO_4$	0.30
KNO_3	0.80
KCl	0.87
KH_2PO_4	0.09
$Fe(SO_4)_3$	0.006

To these salts 2 per cent by weight of sucrose and the filtered extract of 0.01 per cent by weight of dried brewer's yeast were added.

This solution was used in these experiments as given above and designated as solution *C*. Three modifications were used also as follows:

Solution A. As above except that the sugar and yeast extract were omitted.

Solution B. The above solution without sugar but with the yeast extract.

Solution D. The above solution with sugar but without yeast extract and with synthetic hetero-auxin added, 1 part to 20 millions.

The hetero-auxin was very kindly supplied by Professor F. W. Went of the California Institute of Technology.

The solutions were used in liquid form and also with the addition of 1 per cent agar-agar. The agar media were used in Petri dishes and the

cultures in liquid were made in test tubes. Media, tubes and dishes were autoclaved but the tissues were given no sterilization of any kind. Delicate tissues could not be expected to endure treatment with any sterilizing agent and probably would be destroyed by the handling incident to the treatment. Even rinsing with sterile water or with sterile culture solution was omitted because of the injury which might have been caused in the process. The plant fragments were removed in a sterile condition if possible. It is hardly necessary to say that this was not always possible and that any growth of bacteria or fungi on the tissues was invariably fatal to them even though the organisms were merely saprophytic.

Plant Materials.—The use of tissues of embryonic animals by zoölogists led the writer to believe that embryonic plant tissues might prove the most satisfactory subjects for experimentation. Accordingly most of the trials were made with pieces of embryos taken from immature ovules. The choice of species was determined partly by the experience of the author[1] with plants which showed great regenerative capacity and in part, naturally, by the easy availability of the material. In both these respects the common dandelion, Taraxacum officinale Weber, appeared highly desirable. The ox-eye daisy, Chrysanthemum leucanthemum var. pinnatifidum Lecoq. and Lamotte, and wild lettuce, Lactuca canadensis L., were chosen also, and the tomato, Lycopersicon esculentum Mill.

Later in the course of the investigation trials were made with segments of the embryos of Hieracium aurantiacum L., Alyssum saxatile L. var. compactum, Linum perenne L., Plantago major L., Plantago lanceolata L., Malva moschata L., Lathyrus latifolius L., Melilotus alba Desr. and Ligustrum ibota var. regelianum Rehd., but apparently none of these provided satisfactory materials.

In addition to the preceding, meristems from buds of mature plants of Radicula aquatica (Eat.) Robinson, Stellaria media (L). Cyrill. Populus tacamahaca Mill., and Elodea canadensis Michx., were tried.

All cultures were kept in diffuse light in the laboratory and at ordinary room temperature.

Cultures of Segments of Immature Embryos.—Dandelion cultures. Heads of dandelion containing embryos $^1/_2$ to $^3/_4$ grown were flamed and the achenes were removed to sterile slides with flamed forceps. The ovary wall was torn away and the contents removed to another part of the slide. In turn the seed coat was removed and then the endosperm. The embryo was then cut into pieces. The cotyledons were severed at their bases and the hypocotyl was cut in two about midway of its length. These cuts provided one segment composed of the base of the hypocotyl with the radicle attached; one made up of the upper hypocotyl with the plumular meristem and the bases of the cotyledons; and the severed cotyledons themselves. Most of the pieces used were less than 1 mm. long and many

were not over 0.5 mm. in length. Usually they were under 0.5 mm. in diameter. In addition to the sections described some embryos were sliced into only two pieces, with the upper part bearing the cotyledons intact with the plumular meristem between them, and the lower part consisting of a piece of hypocotyl with the radicle at its lower end.

Results.—The cotyledons expanded greatly on agar media, less in liquid media but in all cases failed to root or form buds, a rather unexpected result in view of the large number of cotyledons from seeds of various species which have been rooted by the author.[1]

Radicles on the lower ends of short pieces of hypocotyls on solution *D* agar showed growth and the formation of root hairs in 3 days. In 10 to 12 days roots had grown out, and new buds had been formed on the cut upper ends. In 30 days roots 2.5 cm. long had developed and most of the plants had four or five leaves. At the end of six months some of the plants were still alive and growing in earth. Each had produced seven or eight leaves and appeared entirely normal and capable of continued growth though they were smaller than seedlings of the same age.

Cultures on agar containing solutions *A*, *B* or *C*, although they sometimes showed some increase in size, failed to continue growth and development.

Pieces of hypocotyl with radicles failed to grow in liquid media, and some which had formed roots and root hairs on agar and were then transferred to liquid cultures remained alive for many weeks but failed to make any further growth.

Segments of the upper ends of cotyledons bearing cotyledon bases and plumular meristems regenerated roots on their cut bases nearly as quickly as the roots grew out on the basal pieces of hypocotyls on which the radicles had been formed previous to the dissection of the embryos. Because the bud meristems were intact on these pieces leaves were formed a little earlier than on the basal pieces of hypocotyls which had to regenerate these meristems. These segments grew into normal plants which soon became indistinguishable from those grown from the basal segments. At the end of six months they were still growing in earth and had six to eight leaves each.

Segments of the upper ends of hypocotyls with the cotyledons intact grew rapidly on solution *D* agar at first and the cotyledons expanded rapidly and turned backward. All produced root hairs on the cut ends of the hypocotyls and a few formed roots but for some reason all of them soon lost their chlorophyll and perished. Similar segments on the other agar media and in liquid cultures showed little growth.

Cultures of Ox-Eye Daisy.—Heads of daisy were taken when the ray florets were just beginning to wither. The embryos were dissected out

from pericarps, seed coats and endosperms as were those of the dandelion and they were cut up into similar pieces.

Results.—Basal pieces of hypocotyl with radicles intact, having a total length of 0.5 mm. on agar containing solution *C*, or solution *D*, produced root hairs in two days. In three days the total length of the segments was 3 mm., the greater part of the expansion representing root growth. After ten days in culture buds had regenerated on the cut upper ends of the hypocotyls of most of the segments which then averaged 10 mm. in length. Growth continued regularly and small normal plants were formed which were growing in earth six months later.

Similar segments on agar with solution *A* or solution *B* failed to develop, and none grew in any of the four liquid media.

Pieces of the upper ends of hypocotyls bearing the cut ends of the hypocotyls and the plumular meristems at their upper ends regenerated roots from their cut lower ends in five days. In six days buds grew out from the plumular meristems and the segments had become complete plants of normal appearance. In 30 days these were 4 to 5 cm. long with 8 to 10 roots and 4 to 6 leaves each. Six months later the plants were still growing in earth and appeared capable of development to maturity.

Segments of hypocotyl similar to the preceding ones on solution *A* and solution *B* in agar, and in all the four liquid media failed to grow.

Excised cotyledons expanded on agar media but not in liquid cultures and failed to form roots or buds under any condition.

Cultures of Wild Lettuce.—Ovules of wild lettuce were taken which were just beginning to turn black in which the embryos were $^1/_2$ to $^3/_4$ grown. These embryos were dissected out and cut up into pieces just as those of the dandelion were. In addition, segments were cut out of the middles of hypocotyls so that they consisted entirely of hypocotyledonary tissue and bore neither bud nor root meristems. This species was tried only on agar with solution *D* as a nutrient.

Results.—Hypocotyls, 0.5 mm. long, with bases of cotyledons and plumular meristems at their upper ends formed root hairs on their cut lower ends in five days. In 10 days they had roots 5 mm. long and leaves 2.5 mm. long while the cut bases of the cotyledons had expanded to a length of 3 mm. After 15 days the roots were 17 mm. long, and the first leaves 20 mm. long. By this time the original pieces of hypocotyls averaged 7 mm. in length. In 25 days the largest plant had a root 49 mm. long and a second root 35 mm. long, one leaf 27 mm. long and a second one 10 mm. long. From this stage the plants grew regularly, and were growing in earth 6 months later with extensive root systems and 8 to 10 leaves each.

Short segments, 0.5 mm. long, of the basal ends of hypocotyls with the radicles intact produced root hairs in 3 days and roots 2 mm. long in 4 days. This was a more rapid root growth than that from the pieces of

hypocotyl without radicles and was to be expected since some time was needed for the regeneration of root primordia on the segments without radicles, but later these plants did not show a more rapid root growth than the others. These segments required 17 days to form new buds. Ultimately these segments produced complete plants indistinguishable from those grown from upper parts of hypocotyls. After 6 months they were growing well in earth.

The segments of the central portions of hypocotyls which had neither plumular meristems nor radicles were cut to a length of 0.5 mm. and placed on agar. In spite of their small size and the lack of any preformed meristem these bits grew. One had a root 22 mm. long at the end of 19 days; nearly as long as the roots formed from the radicle tips in the segments described above. At the same time an emerging bud was seen. In this space of time this bit of tissue had formed both a bud and a root meristem. After 25 days this plant had a root 33 mm. long and a first leaf 6 mm. long. This and other plants from similar segments were still alive and vigorous 6 months later.

The excised cotyledons expanded in culture but failed to produce roots or buds.

Cultures of Tomato.—Tomato fruits less than half grown were opened and the immature seeds removed. Seeds older than this offered considerable difficulty because of the toughness of their seed coats, but at this age they are not very difficult to open without injury to the embryos. The embryos were cut up in the same way as those of wild lettuce and placed on solution *D* agar, and in liquid cultures of solution *D*.

Results.—In tomato alone of the plants tested did the cotyledons regenerate. In this species they formed both buds and roots and grew into complete plants on agar with solution *D* as a nutrient. The cotyledons ranged from 0.5 mm. to 1 mm. in length and in mass were probably about equal to the pieces of hypocotyl used in all this series. The plants formed from them appeared capable of continued growth but were all lost because of fungous contamination.

Segments of the upper sections of hypocotyls with cotyledon bases and plumular meristems at their upper ends formed root hairs and roots on the cut ends of the hypocotyls in a few days on agar. Some produced roots in liquid cultures but did not continue to develop. The plants on agar grew normally but were finally killed by fungi.

Basal sections of hypocotyls with radicles formed roots in from 2 to 3 days and buds within 9 to 10 days on agar. The plants thus formed were small but normal. In liquid extensive much-branched roots developed, but no buds ever appeared though the cultures survived for many weeks, and doubtless could have been subcultured according to White's method.

Segments taken from the midsections of hypocotyls without either bud

or root meristems regenerated both buds and roots on agar and formed complete plants. In liquid cultures extensive roots were formed but no buds. In some cultures the upper ends of the hypocotyls extended into the air above the surface of the liquid but even these failed to form buds.

Cultures of Other Species.—Sections of embryos of the 9 other species mentioned as having been cultured failed to produce any growth.

Cultures of Meristems of Buds.—Cultures of buds of Radicula aquatica. This aquatic plant has shown great power of regeneration in both stems and leaves. The leaves fall off the plants at the slightest touch, and produce new plants from their bases within a very short space of time. They will also regenerate new buds on apical segments of leaves and on internodes of stems. Examination showed that the plants which grew out from the bases of detached leaves came from minute buds imbedded in the leaf bases. In these experiments such small basal buds were dissected out from the petioles and from them the meristems were removed and placed on agar containing solution *D*. Others were dropped into solution *D* in test tubes. At the time of their removal the meristems were under 0.5 mm. in length.

Results.—Roots were seen on the agar cultures after 5 days, and after 28 days the best plant had one root 24 mm. long, another 14 mm. long, one leaf 19 mm. long and a second one 5 mm. long. After the expiration of 37 days this plant had 7 leaves and was growing well. It was transferred to earth covered with water and 6 months later bore 12 active leaves as well as several old dead leaves. Several other plants developed a little less rapidly but grew well.

In the liquid solution *D* several plants developed in the same way as those on agar and only a little less rapidly during the first month. The later growth was much less rapid, though several were still alive after 6 months had elapsed.

Cultures of Buds of Stellaria Media.—Very small meristems were removed from buds and placed on solution *D* agar. However, they showed little tendency to grow and soon perished. It is doubted whether any of them were entirely sterile.

Cultures of Buds of Populus tacamahaca.—Meristems were removed from these buds as in the preceding species but showed only slight expansion after several weeks. They were then dying and were discarded.

Cultures of Buds of Elodea Canadensis.—Terminal buds of this species were dissected and the meristems placed on solution *D* agar and in solution *D* in tubes. Most of the meristems were not sterile, and those which appeared sterile made no growth and died after 2 or 3 weeks in culture.

No change in polarity was observed in any of the plant segments grown in these cultures.

Discussion.—After the many unsuccessful attempts extending over a

period of 38 years which have been made to grow tissues of seed plants in culture it is remarkable to note the ease with which such tissues were grown in the course of the present investigation. For, aside from the admittedly great troubles with fungous contamination, these tissues were grown with ease. Furthermore, to one familiar with the regeneration from cuttings of stems, leaves and roots of many plants in which the development of new tissues is often a long, lingering process, the speed with which these very small bits regenerated new meristems was truly remarkable. Even the segments of hypocotyls of wild lettuce and tomato which contained neither bud nor root meritsems were able to form both these meristems anew within a few days. The writer is inclined to believe that one of the chief conditions of success lies in the use of the tissues of embryonic plants which only very recently have developed from masses of undifferentiated cells into embryonic organs. They may be regarded as cell masses which are still in a plastic state and have not yet developed the characteristics of tissues fixed in the patterns of mature organs.

An exception to the use of embryonic tissues is seen in the case of Radicula aquatica but this plant, as has been mentioned, has been found by the author to possess unusual powers of regeneration. Certainly the bud meristems of plants cannot be declared unfit for tissue culture, but in all probability they will prove more difficult subjects than truly embryonic tissues. White secured some growth in bud meristems of Stellaria media, more indeed than is recorded in the attempts made by the writer, but failed to get continued development. The author failed also with bud meristems of Populus tacamahaca and Elodea canadensis. It is probable that the species used in trials of bud meristems will have to be selected very carefully until the technique of such cultures has been improved greatly. For that matter the same thing holds true for embryonic tissues for the author failed to secure growth from embryos of a number of species, some of which might have been expected to grow.

A second condition of success may have been the use of the plant growth hormone, auxin, in the nutrient solutions. Only in the ox-eye daisy was successful growth gained without the use of auxin in the cultures, but even in those trials the media did include cane sugar and yeast extract, both of which are known to contain considerable amounts of auxin. However, the present experiments were not planned as a critical test of the necessity of auxin, and it may be found that the embryonic tissues contain enough growth hormone to initiate their development. Further experiment must decide that question for us. For the present we can only say that the use of auxin appears to have been beneficial.

At this stage of progress it appears possible that the use of nutrient solutions solidified with agar-agar may have been an important factor in the success of the cultures. In only one species of those used in these

experiments was continued development of shoots and roots obtained in liquid media, and that species was Radicula aquatica, an aquatic plant capable of living but not of developing fully on unsubmerged mud. An aquatic would be supposed to be best able to regenerate in a liquid, but it must be noted that the Radicula cultures were made of bud meristems, so that these tissue masses did not regenerate buds in liquid but only roots. The bud meristems did develop shoots and leaves in liquid media but not nearly so rapidly as they did on agar. Root development in liquids was very good in the tomato also but no buds were developed under those conditions. It may be that White's[3] failure to secure bud formation on his tomato roots was due to the exclusive use of liquid media.

At present we are far from the ideal of growing whole plants from single cells of seed plants since the tissue fragments used in these experiments contained scores, perhaps even hundreds, of cells. However, they were smaller than any such segments which have been induced to form whole plants prior to this time.

The smallest tissue fragments used in these trials were under 0.5 mm. in length and less than that in diameter. The writer[1] had succeeded previously in rooting pieces of tomato cotyledon 3 mm. square, but not with smaller segments, and even the 3 mm. pieces failed to produce buds. White's root cultures, which never formed buds, were usually grown from root tips about 5 mm. long, ten times as large as the sections used in these tests. So far success has not been obtained with fragments much less than 0.5 mm. in length, but it is hoped that refinement of technique may constantly reduce the size of the initial segments. There seems to be no real reason for supposing that such masses as were used here represent the smallest unit that can be made to grow and differentiate, though it is possible that single cells may prove too small for continued growth. In these trials the mass of tissue used has been reduced greatly from the sizes previously needed for regeneration, and there seems no reason for not designating these growths as "tissue cultures." These then are believed to be the first successful tissue cultures of seed plants in which complete regeneration has taken place and whole plants have been produced.

The growth of specialized plant tissues such as parenchyma for example would result in tissue cultures in the sense in which the term is used in animal studies, but such cultures in plants are probably impossible as yet. Save in pathological growth, any extensive proliferation of tissue seems always to be followed by differentiation and the formation of root and stem primordia. This strong tendency to organization must be overcome before we can secure true cultures of tissues. When we succeed in doing this we may need to apply another term to such growths as are described in this paper, but at present it seems best to use the expression

"tissue cultures," for all such regeneration of plants from minute bits of plants.

Such cultures as these should prove of great service in the study of various problems in morphogenesis, physiology and pathology.

Summary.—1. Pieces, no more than 0.5 mm. in length, have been cut from immature embryos of dandelion, wild lettuce, ox-eye daisy and tomato and grown into complete plants in culture.

2. Segments of the upper ends of hypocotyls have regenerated roots. Basal segments of hypocotyls with radicles intact have grown new buds on the cut ends of the hypocotyls. Pieces of the midsections of hypocotyls without either plumular meristems or radicles, have produced new buds on their upper ends and new roots at their basal ends.

3. There has been no change in the polarity of the segments of the plants.

4. Cotyledons of tomato excised from immature embryos have been grown into complete plants. In the other species studied cotyledons have failed to regenerate.

5. White's solution, modified by omitting the yeast extract and adding synthetic hetero-auxin, was used in successful cultures of all the species. Only the ox-eye daisy has grown on White's solution with yeast extract, but without hetero-auxin. No tissues have regenerated on media without either the hetero-auxin or yeast extract.

6. The successful cultures have been grown on nutrient media with one per cent agar-agar added. Liquid cultures have produced roots but no buds.

7. Meristems from buds of the aquatic Radicula aquatica have been grown into normal plants.

8. All the plants grown in culture have been found capable of growth in soil.

* Papers from the Department of Botany, University of Michigan, No. 556, reporting work done at the University of Michigan Biological Station.

[1] LaRue, C. D., "Regeneration in Mutilated Seedlings," *Proc. Nat. Acad. Sci.*, **19**, 53–63 (1933).

[2] White, P. R., "Plant Tissue Cultures. The History and Present Status of the Problem," *Arch. exp. Zellf.*, **10**, 501–518 (1931).

[3] White, P. R., "Potentially Unlimited Growth of Excised Tomato Root Tips in a Liquid Medium," *Plant Physiol.*, **9**, 585–600 (1934).

THE MAGNETIC PROPERTIES AND STRUCTURE OF HEMOGLOBIN, OXYHEMOGLOBIN AND CARBONMONOXYHEMOGLOBIN

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Over ninety years ago, on November 8, 1845, Michael Faraday investigated the magnetic properties of dried blood and made a note "Must try recent fluid blood." If he had determined the magnetic susceptibilities of arterial and venous blood, he would have found them to differ by a large amount (as much as twenty per cent for completely oxygenated and completely deoxygenated blood); this discovery without doubt would have excited much interest and would have influenced appreciably the course of research on blood and hemoglobin.[1]

Continuing our investigations of the magnetic properties and structure of hemoglobin and related substances,[2] we have found oxyhemoglobin and carbonmonoxyhemoglobin to contain no unpaired electrons, and ferrohemoglobin (hemoglobin itself) to contain four unpaired electrons per heme. The description of our experiments and the interpretation and discussion of the results are given below.

Note on Nomenclature.—The current nomenclature of hemoglobin and related substances was formulated at a time when precise information about the chemical composition and structure of the substances was not available. Now that some progress has been made in gathering this information, especially in regard to chemical composition, it is possible to revise the nomenclature in such a way as to make the names of substances more descriptive than the older names, without introducing any radical changes. In formulating the following set of names we have profited by the continued advice of Dr. Alfred E. Mirsky.

The names whose use we advocate are given below, followed in some cases by acceptable synonyms. The expressions in parentheses are those whose use we consider to be undesirable.

Heme: an iron-porphyrin complex (generic term, used for either ferroheme or ferriheme).

Ferroheme (reduced heme): a complex of ferrous iron and a porphyrin.

Ferriheme (oxidized heme): a complex of ferric iron and a porphyrin.

Ferriheme chloride, hemin: a compound of ferriheme and chloride ion.

Ferriheme hydroxide, hematin: a compound of ferriheme and hydroxyl ion.

Ferrohemochromogen, hemochromogen: a complex of ferroheme and another substance, or two other substances, having the characteristic hemochromogen spectrum and involving covalent bonds from the iron atom to the porphyrin nitrogen atoms and the attached groups.[2] Individual hemochromogens may be designated by specifying the attached groups, as globin hemochromogen (ferroheme and denatured globin), dicyanide hemochromogen, dipyridine hemochromogen, carbonmonoxyhemochromogen, pyridine carbonmonoxyhemochromogen, etc.

Ferrihemochromogen (parahematin): a compound of ferriheme and another substance or two other substances, involving covalent bonds from the iron atom to the porphyrin nitrogen atoms and the attached groups.[3]

Hemoglobin: a conjugated protein containing heme and native globin (generic term, used for both ferrohemoglobin and ferrihemoglobin and also for closely related substances); specifically, ferrohemoglobin.

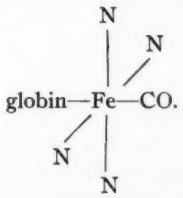
Ferrohemoglobin, hemoglobin (reduced hemoglobin): a conjugated protein formed by combination of ferroheme and native protein.

Oxyhemoglobin: a compound of ferrohemoglobin and oxygen.

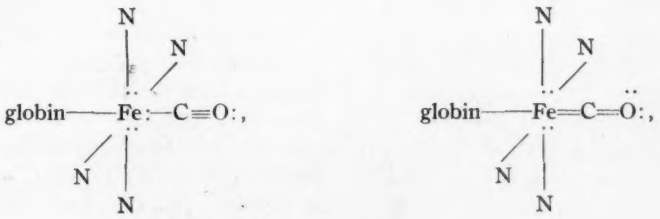
Carbonmonoxyhemoglobin, carbon monoxide hemoglobin (carboxyhemoglobin): a compound of ferrohemoglobin and carbon monoxide.

Ferrihemoglobin (methemoglobin): a conjugated protein formed by combination of ferriheme and native globin.[3]

Carbonmonoxyhemoglobin.—The magnetic measurements are described in the experimental part below. The carbonmonoxyhemoglobin molecule is found to have zero magnetic moment, and hence to contain no unpaired electrons. This is to be interpreted as showing that at least two 3*d* orbitals of each ferrous iron atom are involved in covalent bond formation, the atom presumably forming six octahedral d^2sp^3 bonds, four to the porphyrin nitrogen atoms, one to an atom (probably nitrogen) of the globin, and one to the carbon monoxide molecule:



In view of the discovery of Brockway and Cross[4] that the nickel-carbon bond in nickel carbonyl has a large amount of double bond character, we may well expect this to be the case for the iron-carbon bond in carbonmonoxyhemoglobin also, the double bond being formed with the use of a pair of electrons conventionally assigned to the iron atom as 3*d* electrons. To carbonmonoxyhemoglobin there would then be ascribed the resonating structure:



in which the dashes represent shared electron pairs and the dots unshared electrons.

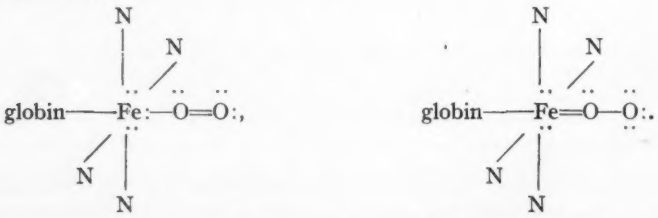
Oxyhemoglobin.—The molecule of oxyhemoglobin, like that of carbonmonoxyhemoglobin, is found to have zero magnetic moment and to contain no unpaired electrons. Each iron atom is accordingly attached to the four porphyrin nitrogen atoms, the globin molecule, and the oxygen molecule by covalent bonds.

The free oxygen molecule in its normal state ($^3\Sigma$) contains two unpaired electrons. It might well have been expected, in view of the ease with which oxygen is attached to and detached from hemoglobin, that the oxygen molecule in oxyhemoglobin would retain these unpaired electrons, a pair of σ electrons of one oxygen atom, unshared in the free molecule, being used for the formation of the bond to hemoglobin:

$$\text{Hb} + :\overset{\cdot\cdot\cdot}{\underset{\cdot\cdot\cdot}{O}}:\overset{\cdot\cdot\cdot}{\underset{\cdot\cdot\cdot}{O}}: \rightleftharpoons \text{Hb}:\overset{\cdot\cdot\cdot}{\underset{\cdot\cdot\cdot}{O}}:\overset{\cdot\cdot\cdot}{\underset{\cdot\cdot\cdot}{O}}:.$$

However, this is shown not to be so by the magnetic data, there being no unpaired electrons in oxyhemoglobin. *The oxygen molecule undergoes a profound change in electronic structure on combination with hemoglobin.*

Of the structures of oxyhemoglobin compatible with the magnetic data, the most probable is the resonating structure analogous to that of carbonmonoxyhemoglobin:



The great similarity in properties of oxyhemoglobin and carbonmonoxyhemoglobin provides strong support for this structure. The structure in which each of the two oxygen atoms (connected with one another by a single bond) is attached to the iron atom by a single bond is rendered improbable by the strain involved in the three-membered ring.

Ferrohemoglobin.—In contrast to oxyhemoglobin and carbonmonoxyhemoglobin, hemoglobin itself contains unpaired electrons, its magnetic susceptibility showing the presence of a pronounced paramagnetic contribution. The interpretation of the magnetic data can be made only in conjunction with a discussion of the nature and magnitude of the mutual interactions of the four hemes in the molecule.[5] One possibility is that the heme-heme interaction is sufficiently strong to couple the moments of all electrons in the molecule into a resultant moment, with the same value for

all molecules. The magnetic data interpreted in this way lead to the value $\mu = 10.92$ Bohr magnetons for the moment of the molecule. We reject this possibility on the following grounds. (1) The heme-heme interaction energy, as evaluated from the oxygen equilibrium data,[5] is hardly large enough to overcome the entropy advantage of independent heme moments. (2) The value 10.92 for the moment is not far from that (8.94) for eight unpaired electrons, two per heme, with parallel spins; however, it is about 22% larger, and this difference could be accounted for only as a surprisingly large contribution of orbital moment. (3) On this basis the magnetic susceptibility of partially oxygenated hemoglobin solutions would show large deviations from a linear dependence on the amount of uncombined heme; we have found large deviations not to occur. (These experiments will be described in a later paper.)

The other simple possibility, which we believe to be approximated in reality, is that the magnetic moments of the four hemes orient themselves in the applied magnetic field independently of one another. With the calculations made on this assumption, the experimental data lead to the value $\mu = 5.46$ Bohr magnetons for the effective moment per heme. This shows that there are present in each heme four unpaired electrons, and that consequently the iron atom is not attached to the four porphyrin nitrogen atoms and the globin molecule by covalent bonds, but is present as a ferrous ion, the bonds to the neighboring atoms being essentially ionic bonds.

The resultant spin moment for four unpaired electrons is 4.90 magnetons. In compounds containing ferrous ion values of 4.9 to 5.4 are observed, the increase over the spin moment arising from a small orbital contribution. Complexes of ferrous iron with substances containing nitrogen (hydrazine, etc.) give values in the lower part of this range, the quenching of orbital moment being nearly complete.[6] It does not seem probable that the high value for ferrohemoglobin is to be accounted for as due to orbital moment, since the porphyrin nitrogen atoms should have a strong quenching effect on the orbital moment. We interpret this high value instead as due to a heme-heme interaction which tends to stabilize states with parallel heme moments relative to those with opposed heme moments, the oxygen-equilibrium value of the heme-heme interaction energy being of the order of magnitude required for this interpretation.

It is interesting and surprising that the hemoglobin molecule undergoes such an extreme structural change on the addition of oxygen or carbon monoxide; in the ferrohemoglobin molecule there are sixteen unpaired electrons and the bonds to iron are ionic, while in oxyhemoglobin and carbonmonoxyhemoglobin there are no unpaired electrons and the bonds are covalent. The change from ionic bonds to covalent bonds also occurs on formation of hemochromogen from ferroheme. Such a difference in bond

type in very closely related substances has been observed so far only in hemoglobin derivatives.

It is not yet possible to discuss the significance of these structural differences in detail, but they are without doubt closely related to and in a sense responsible for the characteristic properties of hemoglobin. For example, the change in multiplicity of the system oxygen molecule–heme in hemoglobin on formation of oxyhemoglobin need be only as great as two (from the triplet corresponding to the opposed oxygen molecule triplet and ferroheme quintet to the singlet of oxyheme), whereas the change in multiplicity on formation of carbonmonoxyhemoglobin is four; in view of the infrequency of transitions involving a change in multiplicity, we might accordingly anticipate that the reactions of hemoglobin with carbon monoxide would be slower than those with oxygen, in agreement with observation. The change in multiplicity may be related also to the photochemical reactivity of carbonmonoxyhemoglobin. The difference in bond type in hemoglobin and its compounds is probably connected with the preferential affinity of hemoglobin for oxygen and carbon monoxide in contrast to other substances. Further experimental information is needed before these questions can be discussed in detail.

Experiments.—Solutions: Defibrinated bovine blood (provided through the courteous coöperation of Cornelius Bros., Ltd.) was used as the source of material. Preparations *A* and *B* consisted of whole blood, collected and separately oxygenated by rotating 20 minutes in air in a large open vessel, and then packed in ice and used as soon as possible. For preparations *C* and *D* oxygenated blood was centrifuged, and the corpuscles washed three times with equal volumes of physiological sodium chloride. Ether was used to hemolyze the collected corpuscles, the stromata-emulsions were separated by centrifuging, and the dissolved ether removed from the oxyhemoglobin solutions by a current of air. The solutions were kept on ice until used.

Analyses were made for oxygen content in a Van Slyke-Neill constant-volume blood gas apparatus. The transfer pipet was calibrated for content and retention on the walls of whole blood or concentrated oxyhemoglobin solution corresponding to conditions of use; the gas pipet was also calibrated for volume. Correction was made for dissolved oxygen on the assumption that the quantity dissolved is proportional to the water present in the solution.

Corrected results of analyses: Blood *A:* 100 ml. combine with 20.20 ml. O_2 S.T.P.; formality of heme-iron, 0.00902. Blood *B:* 100 ml. combine with 20.59 ml. O_2 S.T.P.; formality of heme-iron, 0.00919. Solution *C:* 100 ml. combine with 37.15 ml. O_2 S.T.P.; formality of heme-iron, 0.01658. Solution *D:* 100 ml. combine with 41.26 ml. O_2 S.T.P.; formality of heme-iron, 0.01841.

Apparatus: The apparatus for magnetic susceptibility determinations has already been described.[3] All hemoglobin solutions were measured against water in a tube of about 18 mm. internal diameter. Fields of 7640 and 8830 gauss were used, the forces being reported as average Δw (in milligrams) for the former. A small correction to the observed Δw has been applied for blank on the tube, so that reported forces are for solution against pure water. Solutions were measured at approximately 20°C.

Calibration of field and tube with water against air: $\Delta w = -49.59$. (For hemochromogen and 6*N* NaOH the tube with $\Delta w = -45.40$ for water against air was used.)

Carbonmonoxyhemoglobin.—Samples of blood *A* equilibrated with CO by rotation of 50 ml. in a liter tonometer filled with pure carbon monoxide: $\Delta w = -0.56, -0.60, -0.76, -0.61$, average -0.63. Samples of blood *B* equilibrated with CO: $\Delta w = -0.84, -1.03, -0.80, -0.86$, average -0.88. Samples of solution *C* equilibrated with CO: $\Delta w = -0.28, -0.68$, average -0.48. Samples of solution *D* equilibrated with CO: $\Delta w = -0.36, -0.45$, average, -0.41. (Completeness of saturation with carbon monoxide was generally tested by adding $Na_2S_2O_4 . 2H_2O$ to the magnetic tube and measuring the increase in susceptibility due to formation of hemoglobin.)

We have established in the previous paper[2] the presence of no unpaired electrons in globin hemochromogen and dicyanide hemochromogen. For globin hemochromogen made by denaturing 32 ml. of whole blood with 10 ml. of 6*N* NaOH after reduction of the heme: average $\Delta w = -1.71$; for dicyanide hemochromogen prepared in a similar manner: average $\Delta w = -1.53$; average for the two, $\Delta w = -1.62$. Measurement of the 6*N* NaOH against water in the same tube gives $\Delta w = -4.88, -4.95$. Assuming the additivity of atomic diamagnetism (Wiedemann's rule), whole blood without paramagnetic constituent should give $\Delta w = -0.58$ in the tube used for the hemoglobin series. This value is in satisfactory agreement with the Δw values given above. The calculated value for blood with two unpaired electrons per heme, and independent hemes, is $\Delta w = +1.52$, for four, $\Delta w = +5.69$; the calculated values for the hemoglobin solutions are about twice as great.

Conclusion: carbonmonoxyhemoglobin contains no unpaired electrons.

Oxyhemoglobin.—Samples of blood *A:* $\Delta w = -0.65, -0.40, -0.44$, average, -0.50. Blood *B:* $\Delta w = -0.58, -0.62, -0.62$, average, -0.61. Solution *C:* $\Delta w = -0.44, -0.55, -0.50, -0.50$, average, -0.50. Solution *D:* $\Delta w = -0.38, -0.36$, average, -0.37. Oxyhemoglobin relative to carbonmonoxyhemoglobin: *A*, $+0.13$; *B*, $+0.27$; *C*, $+0.02$; *D*, $+0.04$; calculated for two unpaired electrons on oxyhemoglobin: *A*, $+2.03$; *B*, $+2.07$; *C*, $+3.74$; *D*, $+4.11$.

Conclusion: oxyhemoglobin contains no unpaired electrons.

Hemoglobin.—35 ml. of blood *A* reduced in differential tube by addition of from 0.4 to 1.0 g. $Na_2S_2O_4 . 2H_2O$: $\Delta w = +7.32, 6.85, 6.98, 6.97$, average, $+7.03$. Taking the mean of the oxy- and carbonmonoxyhemoglobin values (-0.57) for Δw of diamagnetism of hemoglobin, the change on removing coördinating group (O_2, CO) is $+7.60$ gm., corresponding to paramagnetism and a magnetic moment of 5.48 Bohr magnetons per heme, assuming independent hemes. (The change in diamagnetism involved in loss of of CO or O_2 is negligible.)

Blood *B*, reduced: $\Delta w = +7.21, 7.16, 7.51, 7.22, 7.50, 7.20, 7.51, 7.06$, average, $+7.30$; diamagnetic value, -0.74; change, $+8.04$; $\mu = 5.58$.

Solution *C*, reduced: $\Delta w = +13.09, 12.83, 12.89, 13.28$, average, 13.02; diamagnetic value, -0.49; change, $+13.52$; $\mu = 5.38$.

Solution *D*, reduced: $\Delta w = +14.95, 14.33$, average, $+14.64$; diamagnetic value, -0.39; change, $+15.03$; $\mu = 5.40$.

Summary of results for hemoglobin: blood *A*, $\mu = 5.48$; blood *B*, 5.58; solution *C*, 5.38; solution *D*, 5.40; average of the four, $\mu = 5.46$.

Spin moment for four unpaired electrons, 4.9; for two, 2.83; for none, 0.00. Moment observed for ferrous ion in solution, about 5.3; moment observed for solid $Fe(N_2H_4)_2Cl_2$, 4.86. Conclusion: ferrohemoglobin has a susceptibility corresponding to four unpaired electrons per heme, with evidence for some magnetic interaction between the hemes.

Summary.—It is shown by magnetic measurements that oxyhemoglobin and carbonmonoxyhemoglobin contain no unpaired electrons; the oxygen molecule, with two unpaired electrons in the free state, accordingly under-

goes a profound change in electronic structure on attachment to hemoglobin. The magnetic susceptibility of hemoglobin itself (ferrohemoglobin) corresponds to an effective magnetic moment of 5.46 Bohr magnetons per heme, calculated for independent hemes. This shows the presence of four unpaired electrons per heme, and indicates that the heme-heme interaction tends to stabilize to some extent the parallel configuration of the moments of the four hemes in the molecule. The bonds from iron to surrounding atoms are ionic in hemoglobin, and covalent in oxyhemoglobin and carbonmonoxyhemoglobin.

We have been helped a great deal by the advice and encouragement of Dr. Alfred E. Mirsky of the Hospital of the Rockefeller Institute. This investigation is part of a program of research on the structure of hemoglobin being carried on with the aid of a grant from the Rockefeller Foundation.

[1] A. Gamgee, *Proc. Roy. Soc. London*, **68**, 503–512 (1901), and one or two more recent investigators have reported blood to be about as diamagnetic as water, without discovering the difference between arterial and venous blood.

[2] L. Pauling and C. D. Coryell, These PROCEEDINGS, **22**, 159 (1936).

[3] We shall discuss the structure of ferrihemoglobin and the ferrihemochromogens in a later paper.

[4] L. O. Brockway and P. C. Cross, *Jour. Chem. Phys.*, **3**, 828 (1935).

[5] L. Pauling, These PROCEEDINGS, **21**, 186 (1935).

[6] L. Pauling, *Jour. Am. Chem. Soc.*, **53**, 1367 (1931).

STRUCTURE AND ARRANGEMENT OF SALIVARY GLAND CHROMOSOMES IN DROSOPHILA SPECIES

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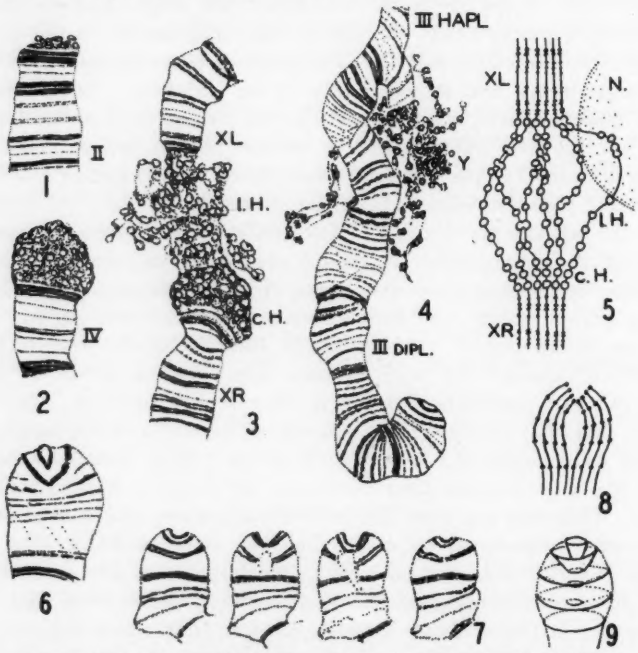
The salivary gland nuclei in Drosophila contain two different chromosome derivatives, the long chromosome strands which correspond to the euchromatic parts of prophase chromosomes, and the chromocenter which arises from the heterochromatic regions (Heitz[2]). While most investigators now agree that the euchromatic strands are composed of a number of closely united chromonemata, the homologous chromomeres of which form discs (aggregate chromomeres), there is no uniformity of opinion regarding the chromocenter. Painter[3] considered it an aggregate of accessory material into which each chromosome sends a thin achromatic strand. Muller and Prokofjeva[4] believe that these regions show the same

regular banding as the euchromatic parts. Koller[5] calls the chromocenter an "undifferentiated magma."

Observations in Chironomidae[6] led me to the conclusion that heterochromatic regions of salivary gland chromosomes are composed of the same number of chromonemata as the euchromatic strands, the difference between them being due to the structure of the single chromomeres. In euchromatic parts, totally staining dot-like euchromomeres are present, while heterochromatic parts are composed of heterochromomeres which are larger in size and stainable only on the periphery. On the basis of these observations it was expected that in Drosophila the chromocenter also is completely chromosomal in nature (not an apposed extraneous material), and that the apparent vacuolization is the expression of a close union and a partial fusion of heterochromomeres.

In the salivary gland nuclei of Drosophila pseudoöbscura the proximal parts of the chromosomes are mostly united into a compound chromocenter. By pressing the preparation it is possible to disrupt the chromocenter and to isolate some chromosomes. In these cases the parts of it are not distributed at random among these isolated chromosomes, as should be expected if it were an amorphous secretion product. Mostly each carries a part of it, constant in amount and structure. Two autosomes (III, IV) possess rather voluminous heterochromatic bases which appear to be formed of a heavily stainable, irregularly vacuolated material (Fig. 2). In some cases these "vacuoles" are arranged rather regularly in discs. That they represent the heterochromomeres, can be seen in more flattened chromosomes in which they are better isolated. The autosomes II and V show only one such heterochromomere discs each (Fig. 1). The chromocenter region of the X-chromosome differs from that of the autosomes in that it forms a looser, net-like body which often covers a part of the nucleolus surface. In contradistinction to the autosomes, the two limbs of the X, if separated by pressure, do not receive a constant amount of the chromocenter material. In most cases the longer limb has the larger part of it. In some nuclei the network forms a communication between both limbs (Fig. 3). The heterochromatic parts of the X consist of the same "vacuolized" granules, the heterochromomeres, as the bases of the autosomes; however, they are not apposed so closely, but form irregular, partly fused strings. Due to this, the heterochromomeres can be more easily recognized as single bodies. Judged from the different amounts of heterochromatin which each limb may receive, there is no predetermined breakage place in the X. Nevertheless, there is some evidence for determining the point of the spindle fibre attachment. The right limb possesses next to the euchromatin a more compact heterochromatic region which is equal in size and structure to the bases of chromosomes III and IV. According to the assumption that the V-shaped X-chromosome

is originally formed by the association of two rod-like chromosomes (the X and an autosome), the spindle fibre is probably located at the transition point between the loose and the compact heterochromatic regions. The only possible structural relation of the euchromatic and the heterochromatic parts is represented diagrammatically in figure 5.



Figures 1–5.—D. pseudoöbscura. Figure 1, proximal end of chromosome II; figure 2, of chromosome IV. Figure 3, proximal part of *X*. Figure 4, III—*Y*-Translocation. Figure 5, diagram corresponding to figure 3; *XR, XL,* right and left limbs of *X*, *C.H.* and *L.H.*, compact and loose heterochromatin. The lines represent the chromonemata. The relation between the loose heterochromatin and the nucleolus (*N*) is indicated. Figures 6–9.—D. hydei. Figure 6, chromosome end with "ring"-bands. Figure 7, optical sections through the end of another chromosome with similar structure. Figure 8, diagram of the course of the chromonemata, corresponding to figure 7. Figure 9, same, showing the form of the terminal chromomere discs. Mag. 1–4: 1830×; 6, 7: 2430×.

The nature of the Y-chromosome in the salivary gland nuclei could be determined in translocations.[7] Figure 4 shows a case of a III-Y-translocation. The Y is completely of the same structure as the loose X-heterochromatin. It possesses no region which corresponds to the compact heterochromatin of *XR*.

In all cases where groups of two or three chromosomes remained together, the size of their common chromocenter was equal to the sum of the amounts present in the isolated chromosomes.

Observations on heterochromatic chromosome parts in mitosis of ganglion cells prove the correctness of the above interpretation. The Y is totally heterochromatic; the left limb of the X is heterochromatic in its proximal third, including the nucleolus-bearing secondary constriction. The right arm of the X and two of the autosomes (III, IV) show short heterochromatic regions, the heteropycnosis being not very conspicuous. The second and the fifth chromosomes seem to be completely euchromatic. (A single heterochromomere pro chromonema, therefore, cannot be detected in mitosis.)

The heterochromomeres as components of the heterochromatic parts of salivary gland chromosomes can be clearly seen in the X-chromosome of D. funebris and in the β-heterochromatin of D. virilis. In D. melanogaster, the maps of Bridges[8] show also that the heterochromatic regions (not designated as such) are formed by vesicular chromomeres. This is especially clear in the case of chromosome II, the continuity of which through the chromocenter could be proved. Here the heterochromomeres are quite regularly arranged in discs, while in the X and in III the omission of some letters indicates that the most proximal parts are more irregularly arranged, as in the species mentioned above. The description of Prokofjeva can be made to agree with that presented here on the assumption that she mistook the chromatic edges of the heterochromomere discs for the real aggregate chromomeres. The same more regular union of the heterochromomeres into discs is the case in the X-chromosome of D. hydei.

In D. melanogaster and D. pseudoöbscura the union of the chromosomes in a common chromocenter is, therefore, an expression of an attraction between the heterochromomeres. As I have pointed out,[6] the explanation of this attraction proposed by Prokofjeva[9] homology between all heterochromomere regions, is not proved by any fact. The difference between the loose and the compact heterochromatin argues still more against this hypothesis. The union of the heterochromatic regions is better regarded as the expression of an attraction between non-homologous parts, characterized by a special kind of chromomeres.

D. funebris und D. hydei show a different behavior. In ganglion cells only the X- and Y-chromosomes are heterochromatic, the Y totally, the X of D. funebris in its proximal half, that of D. hydei in its shorter limb, and in a small area of the other limb adjacent to the spindle fibre constriction. The autosomes of funebris are completely euchromatic; in hydei one pair seems to possess a short heterochromatic (proximal?) end (Heitz[2]). Correspondingly, in the salivary gland nuclei only the X (and probably the Y which could not be identified with certainty) possesses

heterochromomeres, the autosomes appear to be completely euchromatic. In D. hydei the X shows the same behavior, but here also one long and probably the short chromosome contain a short heterochromomere region. Nevertheless, all chromosomes tend to be united by their proximal ends. This union, however, is not so intimate as in the species named above, and can be more easily destroyed by pressure.

The cause of this interchromosomal union of euchromatic parts can be shown by a study of the behavior of the distal free ends of the chromosomes. As I have described in Chironomus Thummi,[10] there exists a tendency of the free ends, to join with each other. In this species only union in pairs occurs in which all ends, except the left one of chromosome IV, take part at random. The same behavior can often be seen in all species of Drosophila. It depends, of course, on the degree of pressure during the preparation. In D. virilis, for instance, in 40 nuclei from a not very strongly pressed slide, 35% of the free ends of the 5 long chromosomes were united in groups of 2–4. Each of them takes part with the same frequency (12–15, $m = 13.6$), showing that the union is not a function of the heterochromomere-like terminal granules, present in the chromosomes I, III and V(=X) (Heitz[11]). The distal ends of the long chromosomes of D. funebris and D. hydei behave in the same manner. This terminal attraction must be effective also in their proximal free ends. The potentially equal attraction of proximal and distal ends to each other can be seen in cases in which chromosomes are only distally united. Chain formations ($Ip = Id - IId = IIp - IIIp = IIId$) occur rather frequently. Rare cases were found, in which the short chromosome was attached to the union point of several distal ends (possibly due to lagging during the last telophase). The difference in amount of proximal and distal union depends probably on the arrangement during the last telophase, in which the proximal ends of all chromosomes come close together, while the position of the distal ends, due to the different length of the chromosomes and the less exact orientation, is more variable.

The terminal attraction can explain two other peculiarities of the salivary chromosomes. The short chromosome in the species so far studied (comp. Bridges,[12] Tan[13]) is mostly curved, both ends being bent together or sticking to the chromocenter. It seems unnecessary to assume with Bridges the possible presence of a distal heterochromatic region in the fourth chromosome of D. melanogaster, as neither of the two criteria for such regions (heteropycnosis in prophases, heterochromomeres in salivary glands) agrees with this possibility. As all species show the same feature, it seems to be only a consequence of the small length of these chromosomes, not of their special structure. This would be expected, if the bending depends on the non-specific affinity between free ends.

The second fact, explainable by the terminal attraction, is the shape of

some chromosome ends. These often show ring-like terminal bands (Fig. 6). Heitz[11] took such figures as an argument for the peripheral arrangement of the chromonemata along an achromatic axis. Optical sections through these chromosome ends (Fig. 7), however, demonstrate that these "rings" can be followed through the interior of the chromosome. They form the edges of discs which are transformed from even plates into funnel-like structures. The course of the chromonemata must be as indicated in figure 8, the shape of the subterminal discs is represented diagrammatically in figure 9. The resulting intrachromosomal cleft can always be seen in such chromosome ends. Sometimes it opens out, especially in cases of excentric arrangement of the discs. These configurations can be understood on the assumption that the terminal chromomeres do not pair laterally, but tend to unite endwise. Simple mechanical conditions prevent the majority of the chromomeres from becoming arranged in this way. The result of this interaction is the special form of the terminal discs.[14] That only some chromosomes show such figures may be due to a competition between the lateral pairing attraction and the terminal affinity.

The special property of free ends is a fact. As an explanation for it, it may be assumed that the terminal chromomeres (or genes) are potentially bipolar but structurally connected only at one pole. The residual force of the free pole tends to become "saturated," which is possible only by union with another free pole (comp. Kossikov and Muller[15]).

Interchromosomal connections thus depend on two different forces: (1) the non-homologous attraction of heterochromomeres, (2) the non-specific affinity of terminal chromomeres. The first is effective in proximally heterochromatic V-shaped chromosomes of Drosophila (but not in the equally shaped chromosomes of Glyptotendipes[6]), the second in euchromatic ends. Both probably act together in the proximal union of partly heterochromatic rods. These forces tend to preserve the general telophase arrangement of the chromosomes.[16]

Painter[3] has raised the further question, whether there is a constant orientation of the chromosomes in regard to each other. He deduces this from the grouping in pairs of the chromosomes after breakage of the chromocenter. He finds that the fourth chromosome remains preferably together with an arm of the chromosome III. These observations, however, cannot decide the question, as the chromosome IV has its constant place in the center of the metaphase plate and consequently of the telophase group, preserved in the salivary gland nuclei. In D. pseudoobscura similar observations show no preferential grouping in pairs. Among 78 isolated pairs the 10 possible combinations of the 5 chromosomes occur with approximately equal frequencies (5–7–8–11; $m = 7.8$).

These results will be published in more detail elsewhere.

[1] Intern. Research Fellow of the Rockefeller Foundation.

[2] Heitz, E., *Zeits. Zellf. mikr. Anat.*, **19**, 720–742; **20**, 237–287 (1933).

[3] Painter, T. S., *Genetics*, **20**, 301–326 (1935).

[4] Muller, H. J., and Prokofjeva, A., *C. R. Acad. Sci. U. S. S. R.*, **1**, 658–660 (1935); Prokofjeva, A., *Ibid.*, **4** (1935).

[5] Koller, P. C., *Proc. Roy. Soc.*, **B118**, 371–397 (1935).

[6] Bauer, H., *Zool. Jahrb. Abt. allg. Zool.*, **56**, 239–276 (in press, 1936).

[7] Mr. C. C. Tan kindly supplied the stocks.

[8] Bridges, C. B., *Jour. Hered.*, **26**, 60–64 (1935).

[9] Prokofjeva, A., *C. R. Acad. Sci. U. S. S. R.*, **2**, 499 (1935).

[10] Bauer, H., *Zeits. Zellf, mikr. Anat.*, **23**, 280–313 (1935).

[11] Heitz, E., *Biol. Zentralbl.*, **54**, 588–609 (1934).

[12] Bridges, C. B., *Trans. Dynam. Development*, **10**, 463–473 (1935).

[13] Tan, C. C., *Proc. Nat. Acad. Sci.*, **21**, 200–202 (1935).

[14] These observations prove that in Drosophila as in Chironomus[10] the chromonemata fill the whole chromosome and are not only peripherally arranged. The second argument of Heitz[11] in favor of the latter view is based on so-called lateral attachments. Configurations like those described by Heitz are present also in D. funebris, D. hydei and D. pseudoöbscura. They represent short duplications (repeats). The rings figured by Heitz are only seldom to be seen and represent edges of the bent discs, as in the chromosome ends.

[15] Kossikov, K. V., and Muller, H. J., *Jour. Hered.*, **26**, 305–317 (1935).

[16] The presence of the terminal attraction accounts partly for the erroneous interpretation of a continuous spireme in early mitosis and meiosis. In the later stages of meiosis it manifests itself after the terminalization of the chiasmata in holding the dyads together.

FURTHER DATA ON LINKAGE IN RABBITS

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Castle and Nachtsheim[1] have shown that two genes (r_1 and r_2) for rex (short) coat in the rabbit are borne in a common chromosome, since they are linked with each other. An estimate was made by them of the crossover percentage based on breeding tests of 51 F_2 individuals derived from a repulsion cross, which indicated that the crossover percentage was about 10 or 12. Subsequently a race of homozygous double recessive individuals ($r_1r_1r_2r_2$) has been established by Castle. These produce only short-haired (rex) offspring when mated either with pure r_1 or with pure r_2 individuals. But if they are mated with F_1 individuals produced by a cross between pure r_1 and pure r_2 individuals, which F_1 animals themselves have a normal coat, a back-cross test for linkage is obtained which should give a more reliable estimate of the crossover percentage than an F_2 population of like size.

In such a backcross all gametes of the double recessive parent are r_1r_2 in constitution. Those of the F_1 doubly heterozygous individuals are of four sorts, the non-crossover classes being r_1R_2 and R_1r_2, respectively, and the crossover classes R_1R_2 and r_1r_2, respectively. Only short-haired (rex) young will result from union of a double recessive gamete (r_1r_2) with either sort of non-crossover gamete, or with the r_1r_2 class of crossover gametes. But normal individuals (not short-haired) will result from the union of a crossover gamete, R_1R_2, with a double recessive gamete, since neither rex gene will then be homozygous. Accordingly, the number of normal individuals obtained in a back-cross population will be equal to half the total number of crossovers, provided the two classes of crossover gametes are produced in like frequency, as they theoretically should be produced.

A back-cross population of 384 individuals has now been produced by mating doubly heterozygous F_1 females with double recessive males. Of the 384 individuals, 33 were normal, the remainder being short-haired.

TABLE 1

PHENOTYPES	♀ F_1	♂ F_1	TOTAL	
Himalayan, black, white fat	229	47	276	Non-crossovers
Himalayan, black, yellow fat	6	1	7	Double crossovers
Himalayan, brown, white fat	109	16	125	Single, region 2
Himalayan, brown, yellow fat	43	3	46	Single, region 1
Colored, black, white fat	54	1	55	Single, region 1
Colored, black, yellow fat	100	8	108	Single, region 2
Colored, brown, white fat	12	4	16	Double crossovers
Colored, brown, yellow fat	246	29	275	Non-crossovers
Totals	799	109	908	

If 33 equals half the number of crossovers, the total number will be 66, which is 17.2 per cent of the entire population of 384 individuals. This, it will be noted, is the indicated crossover percentage in female F_1 individuals. In males it would probably be somewhat smaller.

Castle, in a previous paper,[2] has reported on the crossover percentages observed in a three point cross involving the mutant genes c^H (Himalayan albinism), y (yellow fat) and b (brown coat pigment). These genes occur in a common chromosome in the order named. Crossover percentages reported previously were based on a backcross population of 477 individuals, and the observed cross-over percentages were: Between C and Y, 14.4 ± 1.0 per cent; between Y and B, 26.8 ± 1.4 per cent; and between C and B, 35.8 ± 0.8 per cent. The back-cross population has now been increased to 908 individuals, thus supplying more reliable estimates of the crossover percentages. Data for F_1 male and for F_1 female parents will be presented separately. Eight phenotypes resulted from the backcross of a triple heterozygote to a triple recessive, as shown in table 1.

The order of the genes being *CYB*, it will be convenient to designate the region *CY* as region 1 and the region *YB* as region 2. Accordingly, the total number of known crossovers occurring in region 1 will equal the sum of the two "single" classes indicated in table 1 as occurring in region 1, plus the number of double crossovers (which involve crossing over simultaneously in region 1 and in region 2). Similarly, the total number of crossovers in region 2 will equal the sum of the two classes of single crossovers in region 2, plus the double crossovers. By calculations of this sort, we reach the conclusions indicated in table 2.

TABLE 2

	♀ F_1	PER CENT	♂ F_1	PER CENT	BOTH	PER CENT
Crossovers, region 1	115	14.4 ± 0.8	9	8.25 ± 1.7	124	13.6 ± 0.7
Crossovers, region 2	227	28.4 ± 1.0	29	26.60 ± 2.8	256	28.2 ± 1.0
Crossovers, both regions	342	42.8	38	34.85	380	41.8
Double crossovers	18	2.25 ± 0.3	5	4.58 ± 1.3	23	2.53 ± 0.3
Expected double cr.		4.08		2.19		3.83

It is probable but not certain that crossing-over occurs more frequently in F_1 females than in F_1 males. Table 2 indicates that this is true both for region 1 and for region 2, but the difference is of statistical significance only for region 1 (difference in per cent equals 6.1 ± 1.9). It is known that such a difference between the sexes as regards frequency of crossing-over occurs in mice and rats, and the result of this experiment would seem to indicate that the same is true for rabbits.

The occurrence of interference, that is, of fewer double crossovers than expected on the basis of statistical probability, is indicated in the larger population produced by F_1 females, and in the combined population produced by both sexes, though not by the smaller population produced by F_1 males.

On the basis of table 2 it would appear that the map distance for region 1 is only about half as great as that for region 2. The apparent crossing-over between the genes *C* and *B*, if the variation in fat color is disregarded, is for F_1 females 38.3 per cent, and for F_1 males 25.7 per cent, again an indication that crossing-over occurs more frequently in females than in males. But if we add to the number of crossovers known to have occurred in region 1 the number known to have occurred in region 2, the totals in per cent are for females 42.8 and for males 34.8, which are considerably higher than the percentages obtained when fat color is ignored. Such a difference, as is well known, is a consequence of double crossing-over; that is, of the occurrence of crossing-over simultaneously in region 1 and in region 2, which makes genes *C* and *B* retain their original relation to each other, a segment of chromosome containing the *Y* gene being removed from between them in double crossing-over and replaced by a similar segment from the synaptic mate.

Summary.—1. From a study of a backcross population of 384 individuals the crossover percentage in F_1 females between the two linked genes, r_1 and r_2, for rex (short coat) is estimated at 17.2 ± 0.4.

2. From a study of backcross populations in the production of which one parent was a triple heterozygote and the other a triple recessive for three linked genes (albinism, yellow fat and brown pigmentation), the following conclusions may be drawn:

(*a*) The order of the genetic loci is *CYB*.

(*b*) In region 1 (*CY*) the crossover percentage in females is 14.4 ± 0.8, in males 8.2 ± 1.7; in region 2 (*YB*) the crossover percentage in females is 28.4 ± 1.0, in males 26.6 ± 2.8. The indicated map distance, accordingly, between *C* and *B* is in females 42.8, in males 34.8, crossing-over occurring more freely in females than in males, as is true also in rats and mice.

(*c*) Crossing-over in one region of a chromosome "interferes" with crossing-over in another region, as in *Drosophila*.

[1] *Proc. Nat. Acad. Sci.*, **19**, 1006 (1933).
[2] *Ibid.*, **19**, 947 (1933).

THE METRIC CHARACTERIZATION OF A CLASS OF SPACES

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Introduction.—An abstract space is defined by means of a set of abstract elements, "point" and a structure. The structure attaches a meaning to the relation "near," applied to two elements of the set. For the abstract spaces considered in this note, the structure is given by attaching to each pair of elements p, q a non-negative real number pq, *independent of the order of the elements*, while $pq = 0$ if and only if $p = q$. Such a space is called *semimetric*, and the number pq is the distance of the elements p, q.

One of the principal aims of an investigation concerning the metrical properties of a given semimetric space S is to characterize the space metrically. By this is meant obtaining conditions, in terms of distance relations which are necessary and sufficient to insure that *any* semimetric space satisfying them may be mapped congruently (i.e., with preservation of distance) upon S.

In this note a class of semimetric spaces satisfying a set of six postulates is defined and the metric characterization of the class obtained. Among those spaces belonging to the class considered are the n-dimensional hyper-

bolic number space and, as a limiting case, the euclidean n-dimensional space. A detailed account of the method employed, which introduces a unifying principle into the investigation of such questions, will be published elsewhere.

1. We denote by $\varphi(x;\ \mu)$ a real, single-valued function depending on the real variable x and the parameter μ, which is monotonically increasing (in the strict sense) and for which $\varphi(0;\ \mu) > 0$. If $p_1, p_2, \ldots, p_m$ are m points of a semimetric space, we denote by $\Lambda_m(p_1, \ldots, p_m;\ \mu)$ the symmetric determinant $\left|\ \varphi(p_ip_j;\ \mu)\ \right|$, $(i, j = 1, 2, \ldots, m)$ (where we suppose $\varphi(x;\ \mu)$ defined for every $x = p_ip_j$, $\alpha \leq \mu \leq \beta$), the value of which is independent of the order of the m points. If, now, n be a preassigned positive integer, and μ_0 is a fixed value of the parameter μ, we mean by the n-dimensional space $\Sigma^{\varphi}_{n,\mu_0}$, *any* semimetric space satisfying the following six postulates:

POSTULATE I. $\Lambda_{n+2} = 0$ *for every set of* $n + 2$ *points.*

POSTULATE II. *There exists at least one set of* $n + 1$ *points for which* $\Lambda_{n+1} \neq 0$.

POSTULATE III. *For each set of* $k + 1$ *points sign* $\Lambda_{k+1} = (-1)^k$ *or* 0.

POSTULATE IV. *If* $p_i, \bar{p}_i$, $(i = 1, 2, \ldots, n + 1)$ *are two congruent sets of* $n + 1$ *points of* $\Sigma^{\varphi}_{n,\mu_0}$ *then to each* x *of* $\Sigma^{\varphi}_{n,\mu_0}$ *there corresponds at least one point* $\bar{x}$ *of* $\Sigma^{\varphi}_{n,\mu_0}$ *such that* $p_1, p_2, \ldots, p_{n+1}, x \approx \bar{p}_1, \bar{p}_2, \ldots, \bar{p}_{n+1}, \bar{x}$.

POSTULATE V. *If* $p_1, \ldots, p_n, p$ *are* $n + 1$ *points for which* $\Lambda_{n+1}(p_1, \ldots, p_n, p;\ \mu_0) \neq 0$, *then* $\Sigma^{\varphi}_{n,\mu_0}$ *contains at least one point* $p^0 \neq p$ *such that* $p_1, p_2, \ldots, p_n, p \approx p_1, p_2, \ldots, p_n, p^0$.

POSTULATE VI. *Let* $p_1, \ldots, p_{n-1}, p^*$ *be* n *points for which* $\Lambda_n(p_1, \ldots, p_{n-1}, p^*;\ \mu_0) \neq 0$ *and let* $p^{**} \neq p^*$ *be a point such that* $\Lambda_{n+1}(p_1, \ldots, p_{n-1}, p^*, p^{**};\ \mu_0) = 0$; $p_1, \ldots, p_{n-1}, p^* \approx p_1, \ldots, p_{n-1}, p^{**}$. *Then corresponding to any point* p_n *for which* $\Lambda_{n+1}(p_1, \ldots, p_{n-1}, p_n, p^*;\ \mu_0) = 0$, *and any number* ω *with* $p_np^* < \omega < p_np^{**}$, $\Sigma^{\varphi}_{n,\mu_0}$ *contains at least one point* p_{n+1} *such that* $p_1, \ldots, p_{n-1}, p_{n+1} \approx p_1, \ldots, p_{n-1}, p^*$ *and* $p_np_{n+1} = \omega$.

2. The concepts of congruence order and quasi-congruence order were introduced by Menger in his metrical investigations of euclidean space. A space S has congruence order k provided that any semimetric space, each k points of which is congruent to k points of S, is congruent to a subset of S; while a space S has the quasi-congruence order m if it is such that any semimetric space *containing more than* $m + 1$ *points* is congruent with a subset of S provided each set of m points of the space is congruent with m points of S.

The following theorems are obtained:

THEOREM I. *A semimetric space satisfying* POSTULATES I, II, III, IV *has the congruence order* $n + 3$.

THEOREM II. *A semimetric space satisfying* POSTULATES I–VI *has the quasi-congruence order* $n + 2$.

THEOREM III. *The necessary and sufficient conditions that any semimetric space S be congruent with a subset of* $\Sigma^{\varphi}_{n,\mu_0}$ *are that*

(i) *each two points of S be congruent with two points of* $\Sigma^{\varphi}_{n,\mu_0}$;

(ii) *for each integer* $1 < i \leq n$, *and for every set of* $i + 1$ *points of S the determinant* Λ_{i+1} *formed for the set has the sign* $(-1)^i$ *or* 0;

(iii) $\Lambda_{n+2} = 0$ *for each set of* $n + 2$ *points of S.*

If S contains exactly $n + 3$ *points it is necessary and sufficient to adjoin the condition* $\Lambda_{n+3} = 0$.

Thus, the class of spaces $\Sigma^{\varphi}_{n,\mu_0}$ is characterized metrically.

3. Let $x_1, \ldots, x_n$ be n real variables satisfying the condition $x_1^2 + x_2^2 + \ldots + x_n^2 < \kappa$, $\kappa > 1$. We define a point as the set of n ordered real numbers $(x_1, \ldots, x_n)$, and the distance of two points $x = (x_1, \ldots, x_n)$, $y = (y_1, \ldots, y_n)$ by the expression

$$\text{dist. } (xy) = \frac{\mu_0}{2} \log \frac{1 + \sqrt{1 - \gamma(x, y)}}{1 - \sqrt{1 - \gamma(x, y)}}$$

where

$$\gamma(x, y) = \frac{(\kappa - \sum_1^n x_i^2)(\kappa - \sum_1^n y_i^2)}{[\kappa - \sum_1^n x_i y_i]^2}$$

The relation of μ_0 to κ may be normalized as $\tanh \frac{1}{\mu_0} = \frac{1}{\sqrt{\kappa}}$. This is an n-dimensional hyperbolic number space of curvature $-1/\mu_0^2$. If, now, we let $\varphi(x; \mu_0) = \cosh \frac{x}{\mu_0}$, it is readily verified that this space satisfies the six postulates and is a $\Sigma^{\varphi}_{n,\mu_0}$ space. It is, therefore, characterized metrically by the theorems in §2. It may be noted that in this case condition (i) in Theorem III is automatically satisfied for any semimetric space.

ON THE NON-VANISHING OF CERTAIN FUNCTIONS

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The following theorems are extensions of certain earlier results[1] concerning closure and non-vanishing properties of the set $\{e^{i\lambda_n x}\}$.

THEOREM I. *Let $f(x)\epsilon L(-\pi, \pi)$ and have the Fourier series*

$$f(x) \sim \sum_{-\infty}^{\infty} a_n e^{inx}$$

Let

$$|a_{-n}| \leqslant e^{-\theta(n)}, \quad n = 1, 2, \ldots$$

where $\theta(u)$ is a monotone non-decreasing function such that

$$\int_1^{\infty} \frac{\theta(u)}{u^2} du = \infty.$$

Let $\phi(x)\epsilon L(-\pi, \pi)$ and have the Fourier series

$$\phi(x) \sim \sum_0^{\infty} b_n e^{inx} + \sum_1^{\infty} b_{-n} e^{-i\lambda_n x},$$

where $\lambda_n > 0$ and

$$\lim_{n \to \infty} \frac{n}{\lambda_n} = 0.$$

If $f(x) = \phi(x)$ almost everywhere in some interval then $f(x)$ is equivalent to $\phi(x)$ in $(-\pi, \pi)$.

This is a corollary of

THEOREM II. *Let $\{m_n\}$ be an increasing sequence of positive numbers such that*

$$\lim_{n \to \infty} \frac{n}{m_n} = D, \tag{1.0}$$

and such that for some $\alpha > 0$

$$m_{n+1} - m_n \geq \alpha, \quad n = 1, 2, \ldots.$$

Let $a < \pi D$. If $f(x)\epsilon L(-a, a)$ and

$$\int_{-a}^{a} f(x) e^{im_n x} dx = O(e^{-\theta(n)}) \tag{1.1}$$

where $\theta(u)$ satisfies the same requirements as in Theorem I, *then $f(x)$ is equivalent to zero.*

Proved in the same way as Theorem II is

THEOREM III. *Let $\{m_n\}$ be a positive sequence and let $M(u)$ be the number of $m_n < u$. Let the Polya maximal density*

$$\lim_{\xi \to 1-0} \overline{\lim_{u \to \infty}} \frac{M(u) - M(u\xi)}{u(1-\xi)} = D. \tag{1.2}$$

If we replace (1.1) *by*

$$\int_{-a}^{a} f(x)e^{im_n x}\,dx = O(e^{-\delta m_n})$$

for some $\delta > 0$ then Theorem II *remains valid with* (1.2) *in place of* (1.0).

In proving Theorems II and III we introduce the entire function

$$G(w) = \int_{-a}^{a} f(x)e^{iwx}dx$$

and apply a theorem[2] on the location of the zeros of such functions and then use a basic theorem of Carleman.[3]

A detailed treatment will be given elsewhere.

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[1] N. Levinson, "On the Closure of $\{e^{i\lambda_n x}\}$ and Integral Functions," *Proc. Camb. Phil. Soc.*, **31**, August (1935); and "On a Class of Non-Vanishing Functions," to appear shortly in the *Proc. Lond. Math. Soc.*

[2] N. Levinson, Theorem III, loc. cit., *Proc. Camb. Phil. Soc.*

[3] Titchmarsh, *Theory of Functions*, p. 130.

THE EVALUATION OF ULTRA-VIOLET SOLAR RADIATION OF SHORT WAVE-LENGTHS

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BUREAU OF STANDARDS

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The earliest measurements of the ultra-violet in solar radiation and in artificial sources were undertaken about 8 years ago in connection with an inquiry into the wide-spread interest in light sources and window materials for therapeutic purposes.

From these crude beginnings, as the demand for more exact data increased, improvements were made in the methods of radiometry; including

the development of a balanced thermocouple and filter method which greatly increased the accuracy of the measurements.[1,2]

Throughout these investigations there was an outstanding uncertainty in the measurements, which evidently was attributable to the lack of knowledge of the distribution of energy in the solar ultra-violet spectrum. Recently this uncertainty has been largely eliminated by the use of a photoelectric cell and filter radiometer.[3]

By means of a titanium photoelectric cell (which is insensitive tor adiation of wave-lengths longer than about 3500 Å) and a group of four glass filters, the spectral band of ultra-violet, extending from 2900 Å to about 3500 Å is separated into five parts. This procedure requires only a few minutes, which makes it possible to determine the spectral quality (the spectral energy distribution) of the ultra-violet, of wave-lengths shorter than about 3200 Å, at the time the biological investigations are in progress.

From a study of the shape of the spectral energy curve, required to give calculated transmissions that are in agreement with the observed, photoelectrically integrated, filter transmissions, it appears that a relatively smooth curve with but slight indentations, as observed by Pettit,[4] answers the purpose of evaluating the ultra-violet for use in medicine.

A recent improvement in the experimental procedure is the calibration of the photoelectric ultra-violet meter against a standard of ultra-violet radiation,[5] which makes it possible to determine both the spectral quality, and the total intensity in absolute value, by means of one instrument, thus greatly reducing the equipment, work and expense in conducting field measurements of solar ultra-violet radiation. Preliminary field measurements in the tropics (San Juan, Puerto Rico) and at high elevations (Flagstaff, and the San Francisco Mountains, Arizona; elevation 7300 and 10,500 ft., respectively) have already been made.[6]

In the course of these measurements three distinctly different methods of energy evaluation were used:

(1) The radiometer was a single thermocouple receiver,[1] covered permanently with a cell of water to absorb the infra-red rays, and with a filter of Corning red-purple glass, Corex A, No. 986, to isolate the ultra-violet rays of wave-lengths shorter than about 4000 Å. This combination of filters reduces the intensity to about 8 per cent of the total incident upon the receiver. The apparatus was used also without the Corex glass filter, thus admitting about 75 per cent of the full intensity (the part transmitted by the water cell) upon the thermocouple receiver.

Two exclusion filters of Ba-flint glass were used (singly and in combination) to eliminate some of the uncertainty of the assumed ultra-violet spectral energy distribution (of wave-lengths shorter than 3132 Å), which was based upon the few, highly discordant measurements available. The

radiant flux of ultra-violet, of wave-lengths shorter than and including 3132 Å, was determined in absolute value, in terms of the total incident solar radiation intensity, Q, measured with a pyrheliometer.

(2) A balanced thermocouple and filter radiometer was used,[2] either with or without the red-purple filter of Corex A glass (No. 986). A single exclusion filter of Ba-flint glass (and a balancing filter of clear Corex A glass, No. 980) was used to isolate the ultra-violet of short wave-lengths. This method requires a knowledge of the spectral-energy curve in only the ultra-violet of short wave-lengths, instead of the whole spectrum as used in the preceding method. The radiant flux of ultra-violet, of wave-lengths shorter than and including 3132 Å, was determined in absolute value by calibration of the thermopile against a standard of thermal radiation.[9]

(3) The third and most recently investigated method consists in evaluating the ultra-violet of short wave-lengths by means of a titanium photoelectric cell calibrated, in absolute value, against a standard of ultra-violet radiation.[5]

This method differs also from the two preceding methods in that the solar spectral energy distribution, in the extreme ultra-violet, is determined directly by means of a titanium photoelectric cell and filters, at the time of the ultra-violet radiation measurements, instead of using the highly uncertain extrapolations, previously employed.

Comparative measurements of ultra-violet solar intensities were made also with the balanced thermocouple and exclusion filter of Ba-flint glass, as described in method (2), but employing the directly observed ultra-violet spectral-energy curve in reducing the data. The balanced thermocouple and filter method therefore served as a means of comparing the three methods of evaluating the ultra-violet in sunlight.

The ultra-violet solar intensities, measured by the photoelectric and thermoelectric radiometers, using the ultra-violet spectral energy distributions as observed with the photoelectric cell and filter method, are in good agreement.

Technical details to serve as a guide in evaluating ultra-violet solar radiation of short wave-lengths, by the above-mentioned thermoelectric and photoelectric methods, are described in a forthcoming paper.[7]

Probably the most outstanding result of these studies of various methods of evaluating the ultra-violet, of short wave-lengths in sunlight, is the repeated observation of a radiant flux almost twice as large as the value calculated on the basis of early observations of the ultra-violet spectral-energy curve.

The writers have tried various methods of attack, including the determination of the solar ultra-violet spectral energy distribution. From the close agreement in the data, obtained by three widely different methods of evaluation, it appears that the average intensity of ultra-violet solar radia-

tion, of wave-lengths shorter than and including 3132 Å, during the clearest midsummer weather, at midday, in Washington, is about 75 microwatts per cm.^{2} (μw/cm.^{2}) decreasing to about 8μw/cm.^{2} at the noon hour in midwinter.

Based upon our most recent energy evaluation, a correction is required to the physiological (erythemal) tests of the total ultra-violet solar energy required to produce a minimum perceptible erythema. If such a correction is made the agreement between the observed and calculated erythemogenic efficiency is even closer than previously recorded.[5]

Owing to the smaller amount of ozone in the stratosphere in the tropics, the ultra-violet solar intensities for the same air mass are considerably higher in San Juan than in Washington. For an air mass m = 1.00 to 1.01, which occurs during the noon hour, for about 4 months in San Juan,[6] the ultra-violet intensity, of wave-lengths shorter than and including 3132 Å, is about 95 μw/cm.,^{2} or about 20 per cent higher than is ever attained in Washington (m = 1.05).

The observations at Flagstaff, Arizona, are conspicuous in showing a greater atmospheric transparency (less ozone) in the afternoon than in the forenoon; also a greater transparency in the autumn than in the spring, as previously observed by others. On several occasions there was a temporary increase in atmospheric transparency (increase in the incident ultra-violet radiation); perhaps ascribable to a change in density of ozone, in the stratosphere.

The total ultra-violet intensity at Flagstaff, Arizona, for m = 1.05 is about 115 μw/cm.^{2}, or about 50 per cent higher than observed in Washington. Owing to the more rapid increase in spectral intensity, in the region of 2900 to 2950 Å, which is in the region of the maximum of the spectral erythemic reaction (at about 2967 Å) at the higher altitude, the time to produce a minimum perceptible erythema is reduced to about one-half (9 to 10 minutes) that required at Washington.

On the San Francisco Peaks (Fremont Saddle, elevation 10,500 ft.) intensities of about 155 μw/cm.^{2}, for m = 1.04, and 150 μw/cm.^{2} for m = 1.05 were observed. This is about 35 per cent higher than the maximum observed during the clearest noon hours at the Lowell Observatory station.

Extrapolation of the data observed at the three stations (Washington, San Juan and Flagstaff) indicates an ultra-violet solar intensity of about 600 μw/cm.^{2}, outside the solar atmosphere. This is a 5- to 8-fold increase, as compared with a 20 to 30 per cent increase in the total solar intensity of all wave-lengths.

The spectral intensities of the solar energy curve, as observed with the photoelectric cell and filter radiometer, when extrapolated to zero air mass, show a continuous drop with decrease in wave-length, in good agreement with Pettit's[4] average solar spectral energy curve outside the earth's atmosphere.

A by-product of this research is the opening of a promising field of investigation of stability or homogeneity of ozone in the stratosphere.

In conclusion it is relevant to add that the success attained in depicting the ultra-violet solar spectral energy curve outside the earth's atmosphere, by means of the photoelectric cell and filters, indicates possibilities of obtaining, in a similar manner, the spectral energy distribution of stars, (particularly blue stars) to determine whether there is a continuous drop in intensity with decrease in wave-length, as observed on the sun. A large sized chromaluminized mirror would be required; but since a sharply defined stellar image is not required, the cost of such a mirror would be greatly reduced.

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[2] Coblentz, W. W., Stair, R., and Hogue, J. M., *Ibid.*, **7**, 723–749 (1931).
[3] Coblentz, W. W., and Stair, R., *Ibid.*, **12**, 231–237 (1934).
[4] Pettit, E., *Astrophys. J.*, **75**, 185–221 (1932); *Publ. Astronom. Soc. Pacific*, **47**, 15 (1935).
[5] Coblentz, W. W., and Stair, R., *Jour. Res., N. B. S.*, **16**, 83–92 (1936).
[6] Coblentz, W. W., and Stair, R., *Ibid.*, **15**, 123–150 (1935).
[7] Coblentz, W. W., and Stair, R., *Ibid.*, **16**, April (1936).
[8] Coblentz, W. W., Stair, R., and Hogue, J. M., *Ibid.*, **8**, 759–778 (1932).
[9] Coblentz, W. W., and Stair, R., *Ibid.*, **11**, 79–87 (1933).

THE DOUBLE INNERVATION OF CAUDAL MELANOPHORES IN FUNDULUS

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The support for the thesis that two separate orders of nerves, dispersing and concentrating, are present for the melanophores of Fundulus has come mainly from Smith's (1931) experiments with autonomic depressants and stimulants, and from Mills' (1932) work on background reflexes. Since then it has become customary to speak of the light condition of this fish (induced by the concentration of its cutaneous melanin masses) as due to stimulation of concentrating chromatophore nerves; and conversely, the dark coloration (produced by a dispersion of cutaneous melanin) as one brought about by excitation of dispersing chromatophore nerves. It seems appropriate, in view of recent criticism of the view that melanophores of Fundulus are doubly innervated, to present a preliminary notice of experiments which lend further support to this idea of the pigmento-nervous mechanism of the killifish.

Such evidence was found by studying the responses of melanophores to which nerves were regenerating. In order to prepare specimens for experiments with regenerating nerves, it was first necessary to induce denervated bands of melanophores in the tails of several hundred fishes. Such denervating operations were best made according to the method described by Wyman (1924). After the caudal incisions were made, the animals were returned to a large, illuminated, black aquarium. Within four days after the transection of several radial nerves, the wound region was healed, vascular disturbances were rectified, and the melanophores in the band appeared normal in all respects except that their nervous connections had been destroyed and were degenerating. Both normal and denervated melanophores, when examined, were found to be well expanded due, for the denervated cells, to the combined effects of nerve section and black-background adaptation; and, for the normal cells, merely to the effect of the background. A graphic representation of the conditions of the two sets of cells (normal and denervated) in such fishes is shown in figure 1. The animals were kept in this condition for two or more weeks. During this time the denervated melanophores were incapable of ordinary nervous responses. Placing these specimens on a white background, or stimulating them electrically resulted in the typical contraction of the normal cells but the denervated cells remained fully expanded (Fig. 2). At the end of two or more weeks nerves began to regenerate from their proximal stumps into the denervated area. Methods for ascertaining and measuring this process have already been given. The chromatophore nerves regenerated at the rate of a half millimeter a day; the length of the denervated area was about twelve millimeters; and thus approximately twenty days were open for experimentation with regenerating nerves.

The experimental technique consisted essentially of recording the responses of the same melanophores, which had received regenerated nerve fibres, to a variety of methods of nerve excitation. In order to accumulate reliable data, it was first necessary to find some very rapid method of recording precisely the state of several hundred cells almost instantaneously. Such a method was realized by the use of a Leica camera with a micro-Ibso attachment. The camera was mounted on a microscope equipped with micro-summar lenses and a finely adjustable stage micrometer. A specially constructed glass chamber for holding and spreading the caudal fin was fastened to the stage micrometer. Rapidity of photography was greatly increased by the use of a fast but fine grain moving picture film and a 500-watt source of light filtered through a solution of p. dinitroso-aniline. With this apparatus, the fish could be seized, the same area found repeatedly, and a photograph taken within ten seconds. The enlargements of the negatives proved to be very good data sheets indeed, for the

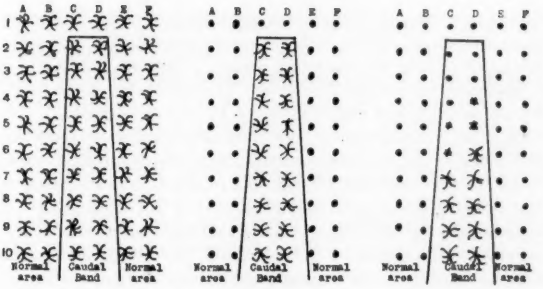

FIGURE 1

FIGURE 2

FIGURE 3

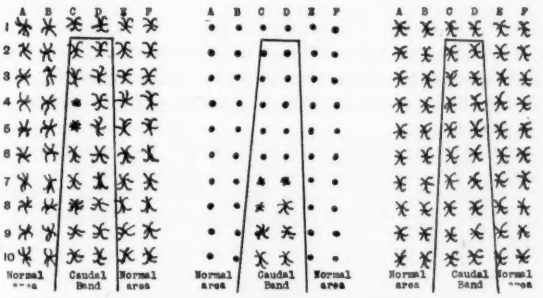

FIGURE 4

FIGURE 5

FIGURE 6

DESCRIPTION OF FIGURES 1 TO 6

All figures are diagrammatic representations of the reactions of melanophores in the same experimental area of a single fish from the time of denervation until almost complete regeneration of nerves to the caudal band. The figures are highly schematized—no attempt is made to represent accurately the form and structure of the individual pigment cells. When expanded (pigment dispersion), these cells are branched, irregularly shaped bodies. When contracted (pigment concentration), they appear as small dots. Intermediate stages also occur. In such stages, the melanophores are more or less stellate. It should also be pointed out that a square millimeter of caudal fin tissue contains around 150 cells. In these figures, however, only a few cells are drawn to illustrate the description of the results.

Figure 1. Condition of normal and denervated cells in the tail fin of Fundulus (black-adapted) following a transverse caudal incision. Both normal and operated melanophores are fully expanded, a condition maintained by keeping the animal on a black background. For a period of two weeks, the denervated band cells are incapable of ordinary nervous responses as figure 2 will show.

Figure 2. Condition of the tail fin melanophores (same area as above) when the animal is white-adapted for ten minutes at any time during a two-week period from the time of operation to the commencement of nerve regeneration. The normal area melanophores contract typically but the caudal band cells remain fully expanded, and are therefore entirely denervated.

Figure 3. Condition of same area 17 days following the operation. The animal was white-adapted for ten minutes. Nerves have now invaded the caudal band as can be

determined by the reactivity of the hitherto unresponsive cells. The normal cells contract typically as do cells 2*C*, 3*C*, 4*C*, 2*D*, 3*D* of the caudal band. These cells are therefore completely innervated by concentrating nerves. Cells 4*D*, 5*D*, 5*C*, 6*C* contract only partly; their innervation is not yet complete. The remainder of the band cells remain fully expanded and are therefore still denervated, or "unregenerated."

Figure 4. Same area ten minutes later during which the animal was black-adapted. Note that cells 4*C* and 5*C* do not expand completely as do the normal area melanophores, band cells 2*C*, 3*C*. 2*D*, 3*D* and cells 4*D*, 5*D*, 6*C*. Cells 4*C* and 5*C* are therefore not innervated at this moment with nerves that produce their dispersed phase. Cells 2*C*, 3*C*, 2*D*, 3*D* are fully reactive by both pigment dispersion and concentration, and behave like normally innervated melanophores. Cells 4*D*, 5*D*, 6*C*, which ten minutes previously (Fig. 3) were unable to contract, are nevertheless capable of pigment dispersion.

Figure 5. Same area three hours later, during which time the animal was kept black-adapted and then placed in a white dish for ten minutes. Note that cells 4*D*, 5*D*, 6*C* which formerly failed to contract completely now do so. Their concentrating innervation is complete. Cell 6*D* which in figure 3 did not respond at all now contracts fully. Cells 7*C*, 7*D*, 8*C* which in figure 3 also did not respond now react partly. Figure 5 compared with figure 3 shows that the zones of reactive, partly reactive and totally inactive melanophores have shifted to the periphery of the tail, a process which goes on at the rate of 0.5 mm. per day.

Figure 6. Same area ten minutes later than figure 5, during which time the animal was black-adapted. All melanophores expand, showing possession of normally dispersing elements. Such a condition after a preliminary ten-minute white background adaptation occurs more often than the condition represented in figure 4 where some melanophores fail to expand.

history of single cells could be followed from one print to another, each taken under different experimental conditions.

The results of this study can be stated most clearly by outlining one of the experimental series. A thoroughly black-adapted fish, two or more weeks after the caudal operation, was examined to insure that the innervated and denervated band melanophores were all well expanded. Figure 1 may serve as an illustration of this condition. The animal was then placed in a white vessel for ten minutes and the typical pallor due to the contraction of all the innervated melanophores (including those of the caudal band which had received regenerated fibres) resulted (Fig. 3). The unregenerated (still denervated) cells of the band remained in a fully dispersed condition. The edge between the regenerated and the unregenerated portions of the originally denervated band was photographed. The animal was then replaced over a black background and after ten minutes the same area was again photographed (Fig. 4). These two photographs were compared and it was sometimes found that not all the melanophores which contracted on a white background expanded on a black one (compare Figs. 3 and 4). Substituting for the ordinary background reflexes mechanical stimulation of nerves (electrical stimulation of the roof of the mouth for contraction of melanophores, and section of the regenerating nerve bundles for the expansion of melanophores) similar

results were obtained. Furthermore, in an area of about fifty cells the following reactions were found:

(1) Some cells were capable of full contraction but not full expansion (cell 4*C*, Figs. 1, 3, 4);

(2) Neighboring cells, situated 50 or more micra away, responding by full pigment dispersion but by incomplete pigment concentration (cells 4*D*, 5*D*, 6*C*, Figs. 1, 3, 4);

(3) Others reacting normally by full expansion and contraction (all cells in normal areas and also cells 1*C*, 2*C*, 1*D*, 2*D*, Figs. 1, 3, 4);

(4) Still other cells responding neither by dispersion nor by concentration of their pigment (cells 6*D*, 7*C*–10*C*, 7*D*–10*D*, Figs. 1, 3, 4).

Such results seem to be in accord only with the idea that these pigmentary effectors are innervated by two sets of nerves, discrete and opposite in action. The use of an animal which serves as its own control is significant, for the end point of melanophore response is judged doubly by the condition of the normally innervated cells and of those innervated by regenerated nerves. Furthermore, only those animals were used which showed a rapid and uniform response throughout body and tail tissues. Thus, when some expanded melanophores do not contract to a white background while all other innervated cells of the tail do, it must mean that those unreactive cells do not have nervous connections, which if properly stimulated, evoke pigment concentration. The proof of this inference was obtained by photographing every half hour the region through which the wave of regenerating nerves was progressing. This was accomplished by white-adapting the same black-adapted fish every thirty minutes and recording the conditions of the cells at the boundary between the reactive and unreactive areas. For as much as three hours, there was no change in the position of this boundary, but at the end of this time it was found that the boundary line had shifted somewhat posteriorly. Compared with photographs taken three hours previously, it was definite that cells, which earlier had failed to respond, were now reacting normally to the white background, or to electrical stimulation (compare Figs. 3, 5, 6; cell 6*D*; also note cells 7*C*, 7*D*). This shifting of the boundary line between the reactive and unreactive cells toward the distal edge of the tail continues steadily, and the length of the area of responsive melanophores gradually increases at the expense of the unreactive portion of the band until finally the entire band is invaded by nerves and therefore becomes fully functional. In the same way, it can be shown that those cells which at one moment contracted fully but failed to expand and vice versa could assume both phases of pigment distribution perfectly normally several hours later when nerves had regenerated completely through that particular region (cells 4*D*, 5*D*, 4*C*, 5*C*, Figs. 3, 4, 5, 6).

Such responses, which go on to completion in one direction and not in

the other for some cells and in the opposite manner for certain others, seem to be explainable best by regarding the concentrated phase of a melanophore to be dependent upon the activity of one type of nerve; and the dispersed phase, upon the activity of another type of nervous connection.

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RELATIONS OF SYMMETRY IN THE DEVELOPING EAR OF AMBLYSTOMA PUNCTATUM

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Read before the Academy, November 19, 1935

The present investigation is an attempt to study the changes underlying embryonic differentiation by means of transplantation experiments in which the orientation of the grafted organ rudiment is varied. The ear was chosen for the purpose, as was the fore limb in a previous study, because it is a paired organ which in its final stage is asymmetric and is the mirror image of the corresponding structure on the opposite side. It has been found that there are definite relations between the orientation of the graft and the character of the resulting organ, particularly in regard to its asymmetry, and that these relations change in regular order with the stage of the embryo at the time of operation (Figs. 5 and 6).

In a previous communication to the Academy[1] it was shown, for instance, that at a certain period of its development, the ear placode, while still an equipotential system in Driesch's sense, behaves as if its anteroposterior axis were polarized and its dorso-ventral not. It was found also that while it is passing into the state of biaxial polarization, which supervenes later, inversion of the dorso-ventral axis of the placode is followed in many cases by a peculiar disturbance characterized by a swollen condition of the resulting ear vesicle. Certain exceptional cases, reported at that time but not included in the brief abstract then published, indicated that in the first period considered the ear rudiment is just emerging from a condition of isotropy in which both axes are indifferent. The experi-

ments were then repeated in the seasons 1925–27 with a more extensive series of stages, and these showed that the condition of isotropy does in fact exist at the earlier stages. Since then Choi[2] has published two studies on the development of the ear in *Bufo japonicus* and *Rana temporaria*, respectively, distinguishing two stages, one (early medullary plate) in which "lateralization" has not taken place, and another (embryo with

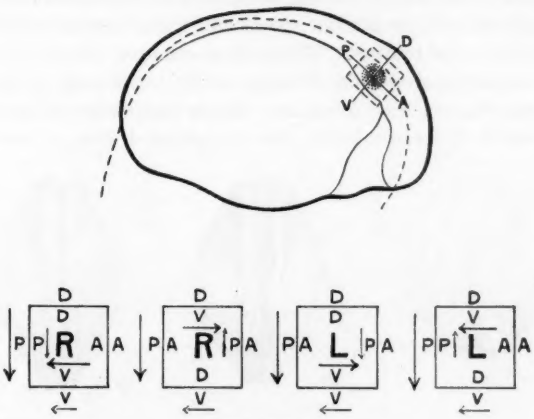


FIGURE 1

Diagram of the operations. The outline of an embryo in profile (Stage 21) is shown above. The curved broken line represents the embryonic axis; the stippled area, the position of the ear placode not yet differentiated; the square area (broken lines), the graft. The four squares below show the position of the axes in the four orientations, the operations being represented as on the right side of the embryo. *R*, graft from the right side; *L*, graft from the left side; *A*, anterior; *P*, posterior; *D*, dorsal; *V*, ventral. The letters inside the squares give the orientation of the graft; those outside the direction of the cardinal points of the embryo. The arrows denote the intensity and direction of the vectorial factors acting to determine the asymmetry of the ear in the second period of axial polarization; thus the first and fourth arrangements give normal ears, the second and third disharmonic ears (left on the right side).

closed neural folds) in which this quality has become fixed. No mention is made of an intermediate stage, and consequently there is no attempt to refer the conditions found to the independent polarization of the two axes. Earlier studies by Spemann[3] and Streeter,[4] as well as those of Ogawa,[5] deal with the last period and are concerned mainly with the question of recovery of posture after rotation.

Embryos of the spotted salamander, *Amblystoma punctatum*, were used in the present experiments. In the order of axial polarization the ear of this form resembles the fore-limb bud.[6] That these two structures should conform to the same rules in this respect is all the more remarkable in view of the radical difference between them as embryonic systems. The limb is a mesenchymatic rudiment, out of which muscles and skeleton are segregated; the ear is an epithelial system, consisting at first of a circular plate of cells, or placode, from which the mature organ develops by invagination and folding. While these changes can be accomplished only by a complicated shifting of cells, roughly speaking, a circular plate becomes transformed into a sphere. From the walls of the latter, by unequal growth and constriction, the complicated form of the labyrinth

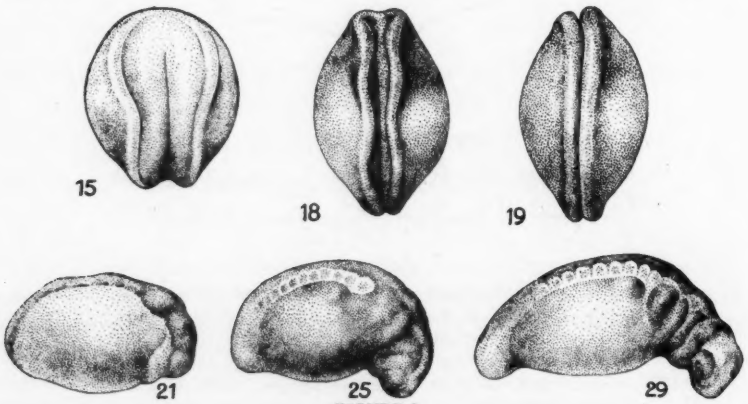


FIGURE 2

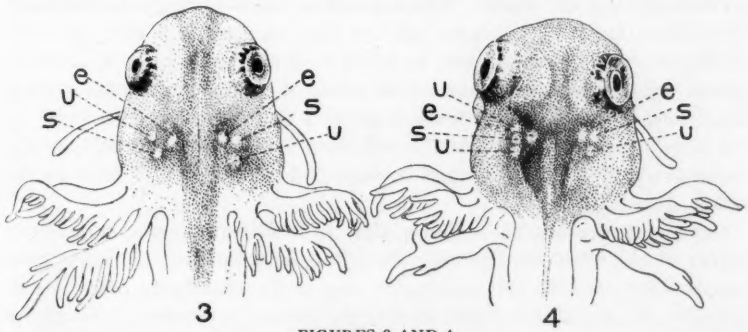
Embryos of Amblystoma punctatum in six of the stages used for operation, the three upper ones viewed from above, the three lower ones from the right side. The numbers denote the stages. × 8.

is evolved. Thanks to the observations of Norris[7] and the experiments of Kaan,[8] the definitive parts of the ear may be projected back upon the circular placode with a certain degree of accuracy. The saccus endolymphaticus and the sensory areas of the saccule arise from the dorsal half of the disc, more particularly from its dorso-posterior quadrant, while the semicircular canals and the utricle are derived from the ventral half.

The area from which the ear develops can be located in the embryo long before visible differentiation occurs. In the orientation experiments a square piece of the ectoderm, including the ear area, was excised and implanted in the same position in another embryo placed in one of four different ways, i.e., either upright or inverted on the same or on the opposite side of the body (Fig. 1). In order that the extent of the graft

might be determined with accuracy at the time when the invaginating ear becomes visible, one of the embryos in each experiment was stained intra vitam with Nile blue sulphate. The graft may be considered as having, besides the axis of rotation, two other axes, an antero-posterior and a dorso-ventral, corresponding to these axes in the embryo. Both may be normally oriented or both inverted, when the graft is placed on the same side from which it came. In grafts to the opposite side, however, one of the axes is alone inverted, while the other remains normally oriented.

Other experiments were made to test equipotentiality and the effect of surrounding structures. The orientation experiments were done at eleven different stages of development, from embryos with neural folds



FIGURES 3 AND 4

Figure 3. Head of larva (Stage 46) showing position of otoliths: *e*, endolymphatic; *u*, utricular; *s*, saccular. Normal condition on left side; on the right a disharmonic left ear which developed after rotation of the right ear placode 180°. Exp. *A V* 156, 26 days after operation. × 10.

Figure 4. Head of larva (Stage 46) with twinned ear (two anterior halves on the left side) which developed following the transplantation of the right half of the hind brain to the left side in antero-posterior orientation. Exp. *MP* 35, 25 days after operation. × 10. Lettering as in figure 3.

just beginning (Stage 15, Fig. 2) to those with moderate tail bud (Stage 29, Fig. 2). In this group 708 cases were brought to maturity, of which 389 have been studied in serial sections. In the remainder, diagnosis of the condition of the ear was made in the living larva either from the position of the otoliths or from the presence of a dome-like prominence in cases of swollen vesicles.

In the normal ear, when illuminated from above, the otoliths appear as three bright spots, forming a triangle (Fig. 3, left side). The brightest, which is nearest the midline, is in the saccus endolymphaticus (*e*); the other two are deeper, one in the utricle (*u*) lying antero-lateral and one in the saccule (*s*) postero-lateral to the endolymphatic. In disharmonic ears (left on right side or vice versa) there is a characteristic arrangement

that occurs in about 85 per cent of the cases; the endolymphatic otolith (*e*) is furthest forward and the other two lie laterally about equidistant from the midline (Fig. 3, right side). However, two such cases out of 26 examined in sections were found associated with ears that were partially twinned. All cases with other arrangements of otoliths have been examined in sections, but no other configuration is closely correlated with any particular kind of ear, except that 60 per cent of the cases composed of two anterior halves have three deep otoliths (Fig. 4, left side), one anterior and two posterior to the endolymphatic.

In the earlier stages the ear rudiment is an "equipotential system"; any part of it can give rise to any part of the definitive organ, depending on its position in the whole. This is shown by the following experimentally determined facts: a fractional part of the rudiment will give rise to a whole ear; two rudiments may be fused to form a single one; a normally placed organ will develop out of an inverted graft; and, under certain conditions, a right ear will develop out of a graft from the left side. As the later stages are approached, localization of the particular parts of the organ gradually takes place and becomes almost irrevocably fixed at the oldest stage used.

Other experiments have shown that in sufficiently early stages any region of the ectoderm has the potency to form an ear, if brought into proper relations with the embryonic axis, or in secondarily induced embryos.[9] At the earliest stages used in the present experiments this power is still largely retained, although the particular area out of which the ear normally develops is already predisposed in this direction.[10,11] Kaan (op. cit.) and Yntema[12] have traced the gradual narrowing down of this quality until, before the older stages used are reached, it becomes restricted to the ear rudiment itself.

When the ear placode, no matter how oriented, is grafted in the medullary plate stage, a normal ear in normal posture develops, indicating that the rudiment is isotropic about an axis perpendicular to its surface, and that the orientation of the definitive ear must be determined by the relations of the rudiment to surrounding structures. There is no evidence of rotation of the graft as a whole, and, moreover, such rotation would not explain the reversal of asymmetry when a graft from one side of the body develops into an ear proper to the other. A few cases show minor defects and there are a few exceptions, referred to below. This isotropic condition continues until the neural folds are high and approaching one another (Stage 19, Fig. 2), at which time a radical change sets in.

During the final closure of the neural folds (Stages 20–21) and for a short time thereafter another condition obtains. The ear placode is no longer isotropic but is polarized in an antero-posterior direction. Transplants taken at this period, no matter how oriented, still develop right

side up, but their antero-posterior differentiation accords with the orientation of the corresponding axis. The result is that normal harmonic ears develop when this axis is normally oriented (Fig. 6), while disharmonic ears develop when it is reversed (Fig. 5), even if the graft is taken from the same side of the body. In other words, at this intermediate period some factor in the surrounding tissues still retains the power to influence the

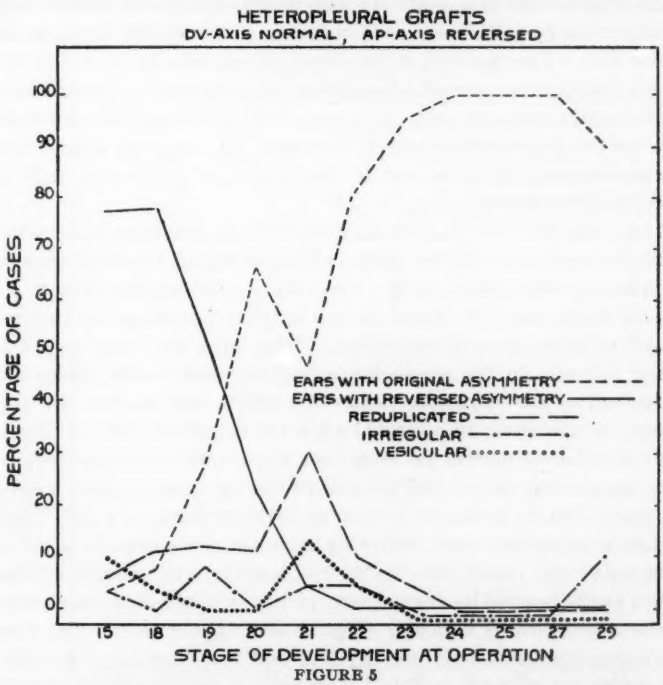


FIGURE 5

Graph showing the behavior of the grafted ear rudiment after reversal of its antero-posterior axis; percentage frequency of each type of result is plotted against stage of development at operation. Ears with original asymmetry are disharmonic, those with reversed asymmetry harmonic. The most marked change takes place from stage 19 to stage 21.

segregation pattern along the dorso-ventral axis, while that of the antero-posterior axis is already fixed in the rudiment itself. The asymmetry of the ear is thus a resultant of two vectorial factors, one in the graft and one in the host (Fig. 1). In this second period the ear placode still remains equipotential.

A few exceptional cases, operated upon in the earlier stages, in which axial relations characteristic of the second period were found, show that

in some few individuals the polarity of the antero-posterior axis is fixed considerably earlier than usual. Viewing the cases as a whole, including those in which both axes of the graft were reversed, the transitional period is seen to be spread over a number of stages, and transplantations made then frequently result in enantiomorphic twinning, or reduplication, of the ear (Fig. 5). This condition is found in 27 per cent of the cases at stage 21 (Fig. 2) and in a relatively high percentage also at stages 19 and 20, which is the period of most sudden change in the lability of the antero-posterior axis. That reversal of the dorso-ventral axis has but little to do with this phenomenon, except when acting synergetically, is shown by the fact that while only two cases (1.4 per cent) of reduplication followed reversal of this axis alone, 21 cases (11.9 per cent) occurred after reversal of the antero-posterior alone and 37 cases (18.6 per cent) when both axes were reversed simultaneously.

The twin ears are not complete in themselves, as double limbs sometimes may be, but are rather of the nature of single organs of which each half is the reflected image of the other. They are of considerable variety. Out of a total of 60 cases, 16 consist of two anterior halves united to form a labyrinth of about normal dimensions. Nine cases are composed of two posterior halves. In the remainder, either the semicircular canals alone are involved in the mirroring, while the utricle and saccule are single (11 cases), or the latter are mirrored while the canals are not (24 cases).

The ear rudiment persists for some time as an equipotential system. At least as late as stage 24 it is still possible to fuse two placodes into a normal single organ, but in grafts made even as early as stage 22 a new kind of disturbance frequently arises, following inversion of the dorso-ventral axis. The primitive otic vesicle fails to constrict itself into its component chambers and swells beyond its normal size, giving evidence of unusual turgor. In these cases, termed vesicular (Figs. 5 and 6), the saccus and ductus endolymphaticus usually do not develop, and if the former is present, it is not connected with the vesicle. This type of disturbance is obviously associated with some change taking place in the direction of the dorso-ventral axis although a few cases, operated upon in earlier stages, in which swollen vesicles have arisen without this axis having been changed, indicate that some other factor may also bring about this condition.

Vesicular ears occur in large numbers after operations done in this second transitional period and at stage 25 (Fig. 2) constitute a majority (57 per cent) of the cases, taking into account inverted grafts from the same side of the body, not shown in the graphs. However, there remain during this period a considerable number of individuals in which the polarity of the dorso-ventral axis is still plainly reversible, for an upright ear may develop after inversion of the rudiment in grafting. This is not due simply to a rotation back to its normal position, as Streeter and Spemann

found after operations at still later stages, since the asymmetry of the organ becomes reversed. This never occurs in ears grafted later than stage 25, after which the dorso-ventral axis must also be regarded as irreversible.

In experiments made upon still older embryos (Stages 27 and 29) a new condition again arises. There is an abrupt decline in the number of vesicular ears and many cases occur in which the ear develops without swelling but in abnormal posture. In some cases it lies with its

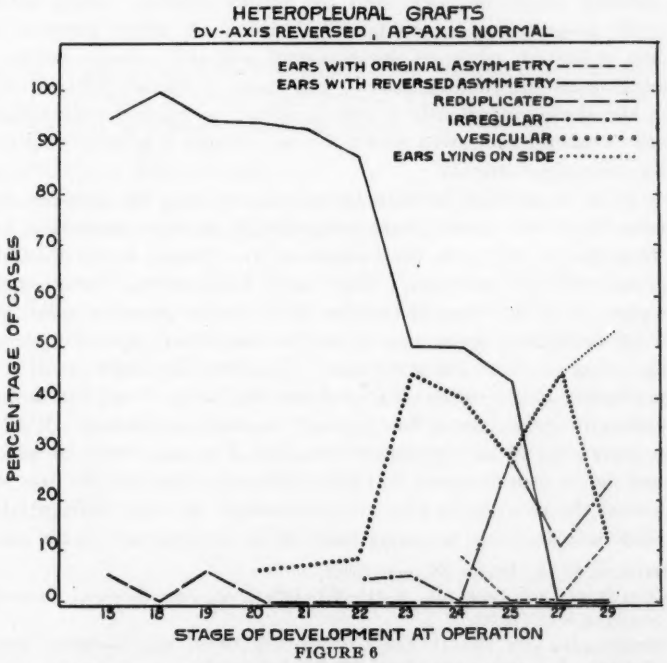


FIGURE 6

Graph similar to the preceding showing results after reversal of the dorso-ventral axis. Period of most marked change between stages 23 and 27.

medial wall ventral, being oriented in accordance with the rotation imparted to the graft, as maintained by Spemann.[3] In other cases it seems to have rotated about its longitudinal axis, sometimes as much as 90°, so as to bring the medial surface further toward the side, with the saccus endolymphaticus lying laterally under the skin—possibly an attempt at regulation, though a mistaken one, since the ear could never attain its normal posture by rotation about this axis.

The fact that the axial relations of the ear are fixed with reference to

their orientation in the embryo leads us to seek the particular agent involved. A series of rotation experiments with the medullary plate (Fig. 4), in which the ear rudiment was left intact, show that it is possible to reverse the polarity of the antero-posterior axis of the ear by this method, although the results are not so constant as when the ear placode is itself rotated.

To sum up the main results of the investigation: The ear rudiment in the neurula stages is isotropic with respect to its differentiation about an axis running perpendicularly from the surface inward. Then, without losing the properties of an equipotential system, its antero-posterior axis becomes polarized, while its dorso-ventral axis still remains subject to influences from surrounding parts. The stage of biaxial polarization, in which the dorso-ventral axis is also involved, is reached concomitantly with the localization of areas within the ear, though it is initiated prior to the loss of equipotentiality.

One is led from these facts to the conclusion that the transformation from the first to the second phase and probably, in some measure at least, that from the second to the third phase involve changes in the orientation of ultramicroscopic elements. That some fundamental transformation takes place in the ectoderm at the time of the antero-posterior polarization of the ear rudiment is shown also by the fact that other important vectorial changes occur at about the same time. It is then that both the direction of the effective stroke of the cilia, as shown by Twitty,[13] and the direction of outgrowth of the lateral line (Stone[14]) become established. With the newer knowledge of the crystalline structure of organic fibres in mind, it may not be too rash to expect that x-ray diffraction photographs may some day reveal the changes in finer structure which underlie differentiations like those just described as taking place in the development of the ear.

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ERRATA

In the "Note on the Effect of Light on the Bioelectric Potentials in the Avena Coleoptile" in these PROCEEDINGS, **21**, December, 1935, pp. 681–684, the following changes should be made:

page 682, Upper graph of figure 1.

1. Illumination should be 800,000 instead of 80,000MCS.
2. The scale of the abscissa should read:

	5,	23,	40,	58,	76,	94,	112 minutes	
instead of,	0,	1/2,	1,	1 1/2,	2,	2 1/2,	3 hours.	

W. G. CLARK